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SOMMAIRE – INHALT – SOMMARIO – CONTENTS

M. STAEHELIN: Chemical Modifications of Virus Infectivity: Reactions of Tobacco Mosaic Virus and its Nucleic Acid 473

Brèves communications - Kurze Mitteilungen - Brevi comunicazioni - Brief Reports

L. CHOLNOKY und J. SZABOLCS: Bemerkungen zur Struktur des Capsorubins 483
M. SHAMMA: The Conformation of 4.5-Substituted Aporphine Alkaloids 484
W. F. BON: Physikalisch-chemische Untersuchungen eines komplexen Eiweisses: das lösliche Eiweiss der Augenlinse, α-Crystallin 485
V. STEFANOVIĆ, A. DJUKANOVIĆ, K. VELAŠEVIĆ, and D. ZIVANOVIĆ: The Investigation of the Action of Ultrasound on some Proteid Substances. Part II. The Action of Ultrasound on Diastase 486
B. RADA, D. BLAŠKOVIČ, F. ŠORM, and J. ŠKODA: The Inhibitory Effect of 6-Azauracil Riboside on the Multiplication of Vaccinia Virus 487
M. KELER-BAČOKA, Z. PUČAR, and A. BENAŠ: A Paper Strip Electrophoretic Examination of the Action of Dextran in Colloidal Solution on Human Serum 488
ANNA CASU: Serotonin Inhibition of Liver Mitochondria Swelling *in vitro* 489
G. KOCH: The Kinetic of Induction of Plaque Formation in Cell Monolayers by Ribonucleic Acid from Poliovirus 490
G. SIEBERT, A. SCHMITT, R. v. MALORTIE und E. ADLOFF: Thermische Denaturierung von Kaltblüter-Enzymproteinen . . . 491
N. J. GIARMAN, L. T. POTTER, and MARGARET DAY: Release of 5-Hydroxytryptamine and Histamine from Neoplastic Mast Cells by Alkaline Tissue Extracts 492
SIMONE HATEM: Histamine et métaux cancérigènes 494
R. GOT et R. BOURRILLON: Action des enzymes sur la gonadotropine urinaire de ménopause 495
S. MALINI: *In vitro* Study of Fe-Cystine Chelate Implicated in Fusariose Wilt of Cotton 496
W. KLAUS, H. LÜLLMANN und E. MUSCHOLL: Der Einfluss von Acetylcholin auf die ^{42}Kalium-Abgabe postnataler, denervierter und reinnervierter Skeletmuskulatur 498
S. RAUCH: Elektrolytgehalt von Endo- und Perilymphe in den einzelnen Schneckenwindungen des Meerschweinchens ohne und mit Stimulation 499
B. S. WOSTMANN and P. L. KNIGHT: The Effect of Methyl Alcohol on the Conversion of Thiamine to Thiochrome 500
R. P. WILLIAMS and K.-C. CHAO: Occurrence of Soluble Pigments in the Genus *Bacillus* 501
J. AXELROD: Enzymatic Conversion of Metanephrine to Normetanephrine 502
M. N. GREEN, G. YERGANIAN, and H. MEIER: Elevated α-2-Serum Proteins as a Possible Genetic Marker in Spontaneous Hereditary Diabetes mellitus of the Chinese Hamster (Cricetulus griseus) 503
D. S. BHATE, R. K. HULYALKAR, and S. K. MENON: Isolation of *iso*-Butyropyrrothine along with Thiolutin and Aureothricin from a *Streptomyces* sp. 504
W. G. JAFFÉ und DINAH S. DE SEIDL: Über die Hemmung von Hurain, einer pflanzlichen Protease, durch Trypsininhibitoren 505
V. ERSPAMER, A. GLÄSSER, and P. MANTEGAZZINI: Pharmacological Actions of 4-Hydroxytryptamine and 4-Hydroxytryptophan 505
V. ERSPAMER, A. GLÄSSER, B. M. NOBILI, and C. PASINI: The Fate of 4-Hydroxytryptophan in the Rat Organism 506
A. STOLK: Induction of Hepatic Cirrhosis in *Iguana iguana* by 3-Monohydroxycholanic Acid Treatment 507
A. STOLK: Changes in the Topographical Distribution of Glycogen in the Brain of *Iguana iguana* in Dependence on the Environmental Temperature 508
V. SPUHLER und K. MEYER: Einwirkung einiger Carbonylreagentien auf das Virus der Maul- und Klauenseuche (MKS) . 509
U. M. MARINARI e G. NANNI: Glicolisi anaerobia del pancreas di cavia 510
R. ZAHRADNÍC and M. CHVAPIL: Study of the Relationship between the Magnitude of Biological Activity and the Structure of Aliphatic Compounds 511
S. GH. NICOLAU et AL. BADANOIU: L'action empêchante de la γ-globuline standard sur le développement de l'infection syphilitique expérimentale chez le lapin 512
F. ROSSI and M. ZATTI: Activation of Fatty Acids in the Liver in Carbon Tetrachloride Poisoning 513
Z. LOJDA and V. FELT: The Histochemistry of the Dehydrogenase Systems in the Aortae of Rabbits with Experimental Atherosclerosis 514
S. ALBERT: The Effect of Litter Rank on the Development of Mouse Lymph Nodes 515
D. M. S. WHEELER and J. W. HUFFMAN: The Stereochemistry of Reduction by Complex Metal Hydrides. (Disputandum) . 516
J. STERZL: Enhancement of Antibody Formation by Hypersensitivity Reaction 518
B. PILLAT and P. HEISTRACHER: Ein einfaches Verfahren zur Sichtbarmachung von Glasmikroelektroden mit Hilfe von Fluorescein (Pro Experimentis) 519
Congressus 520

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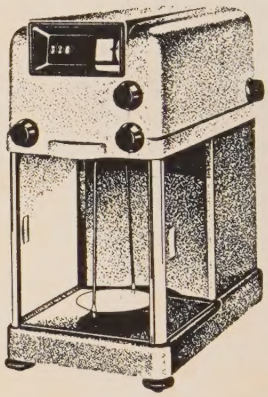

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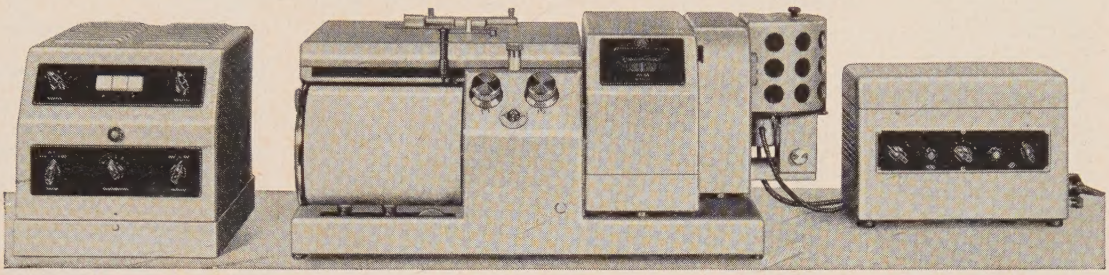

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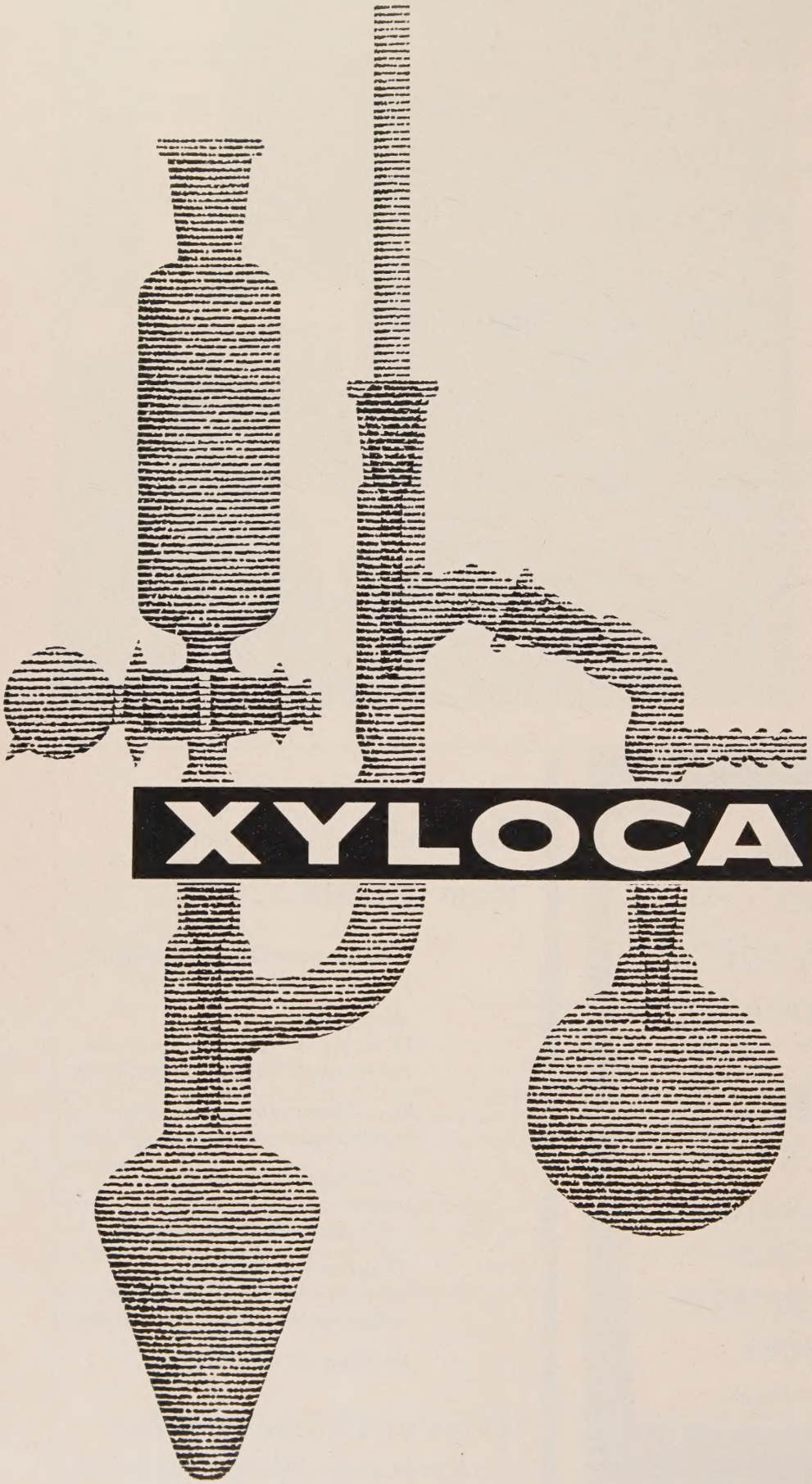

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Chemical Modifications of Virus infectivity: Reactions of Tobacco Mosaic Virus and its Nucleic Acid

By M. STAEHELIN[1]

In many discoveries concerning viruses in general, the tobacco mosaic virus has been a forerunner. It was the first virus ever to be crystallized (STANLEY[2]), but later the crystallization of many other viruses, including animal viruses such as poliomyelitis and Coxsackie virus, have also been achieved (SCHAFFER and SCHWERDT[3], MATTERN[4]). The tobacco mosaic virus was furthermore the first virus whose composition of two components, namely its protein and its ribonucleic acid, has been demonstrated (BAWDEN and PIRIE[5], STANLEY[6]) while later it was recognized that many viruses are built of the same two constituents. And, furthermore, it was the nucleic acid of the tobacco mosaic virus which was first found to be infective. This discovery has to be attributed to GIERER and SCHRAMM[7], as well as to FRAENKEL-CONRAT[8], who were able to demonstrate that the tobacco mosaic virus can be split into its two components and that the isolated ribonucleic acid still carries the infectivity. Many other infective ribonucleic acids from a variety of plant and animal viruses have since been found (COLTER[9]) and it seems a fairly well established fact now that the ribonucleic acids represent the infective part of viruses in general.

The tobacco mosaic virus, therefore, seems to be representative of many viruses. Since it is the virus whose chemical structure is best known, and since it can be obtained in large quantities and is absolutely harmless for the experimentator, it might be justified to use it as a model in order to study some characteristic reactions of viruses in general. In the present study it was used to obtain information on the mechanism of some of the chemical processes which lead to an alteration of the biological properties of viruses.

Structure of the tobacco mosaic virus. The tobacco mosaic virus is a rod about 300 μm long and 15 μm wide (Fig. 1). It grows in such large quantities in infected tobacco plants that over 1 g of pure virus can be obtained from 1 l of sap from infected leaves. It can easily be purified by differential centrifugation from dilute salt solutions. It can also be crystallized by the addition of ammonium sulfate. It is very stable in solution and can be kept for years.

Chemically the tobacco mosaic virus (TMV) consists of about 95% protein and 5% ribonucleic acid. The protein part is made up of a large number of probably identical subunits with a molecular weight of about 18000. Since the TMV particle has a molecular weight of around 4×10^7, there are about 2000 protein subunits present in each virus particle. The nucleic acid molecule, by contrast, is much larger than the protein subunits. GIERER[10] and BOEDTKER[11] have presented physico-chemical and biological evidence indicating that the nucleic acid has a molecular weight of around 2×10^6. This would mean that the whole nucleic acid of a virus particle is contained in one single molecule which consists of about 6000 nucleotides.

The existence of a single polynucleotide chain of 6000 units per particle is not a particular feature of the tobacco mosaic virus but of a large number of viruses. FRISCH-NIGGEMEYER[12] has determined the absolute amount of ribonucleic acid in a variety of viruses of different size and shape and has found it to be a constant value of about 2×10^6, whether the whole virus has

[1] From the Virus Laboratory, University of California, Berkeley (Calif.). Present address: CIBA Limited, Basle (Switzerland).

[2] W. M. STANLEY, Science *81*, 644 (1935); Phytopathology *26*, 305 (1936).

[3] F. C. SCHAFFER and C. E. SCHWERDT, Proc. nat. Acad. Sci., Wash., *41*, 1020 (1955).

[4] C. F. T. MATTERN and H. G. DU BUY, Science *123*, 1037 (1956).

[5] F. C. BAWDEN and N. W. PIRIE, Proc. roy. Soc., London, [B] *123*, 274 (1937).

[6] W. M. STANLEY, J. biol. Chem. *117*, 325 (1937).

[7] A. GIERER and G. SCHRAMM, Nature, Lond., *177*, 702 (1956).

[8] H. FRAENKEL-CONRAT, J. Amer. chem. Soc. *78*, 882 (1956).

[9] J. S. COLTER, Progr. med. Virol. *1*, 1 (1958).

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[11] H. BOEDTKER, Biochim. biophys. Acta *32*, 519 (1959).

[12] W. FRISCH-NIGGEMEYER, Nature, Lond. *178*, 307 (1956).

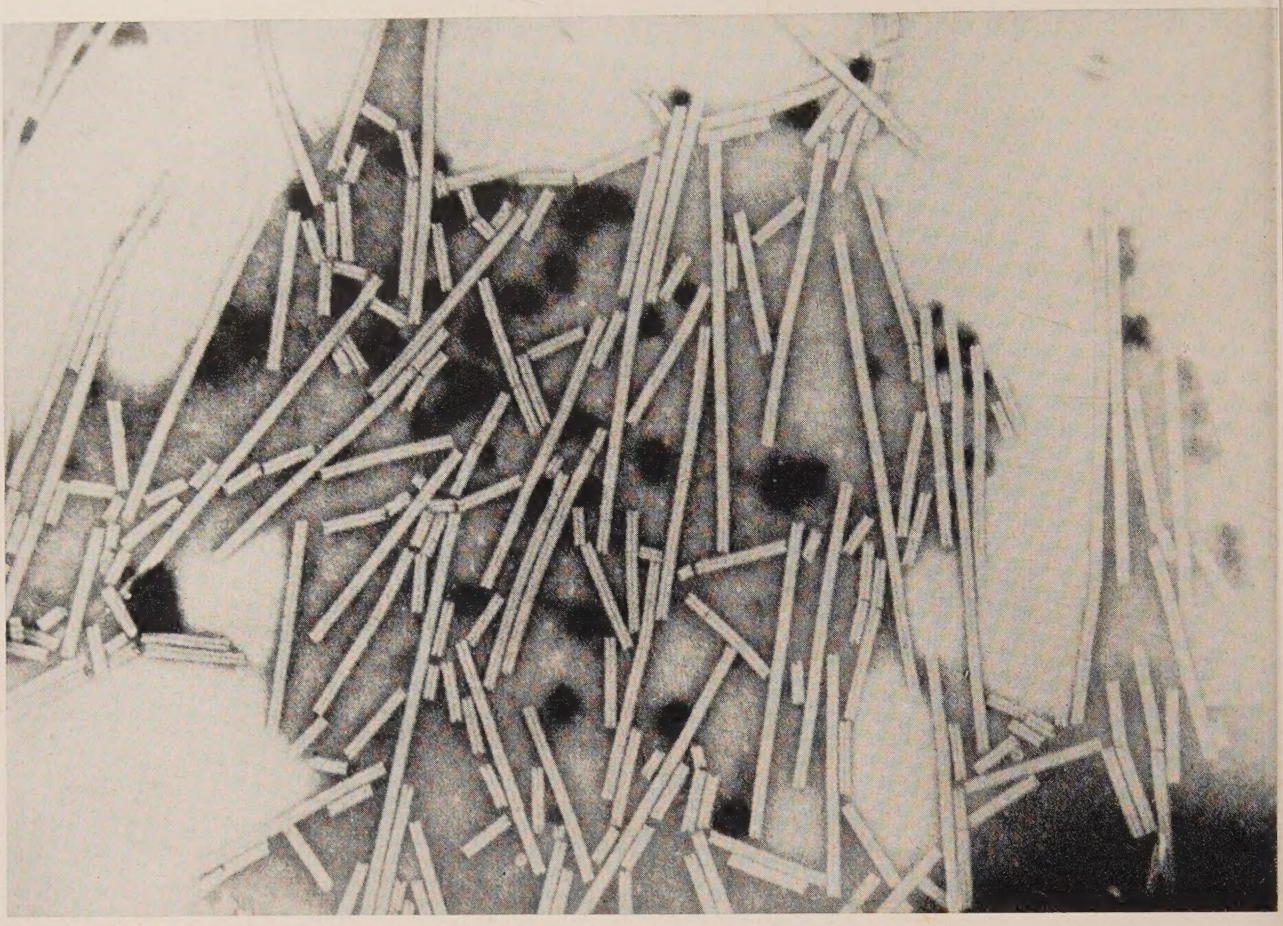

Fig. 1. Electron micrograph of tobacco mosaic virus (Electron micrograph by G. J. HILLS, courtesy of Dr. K. M. SMITH, Cambridge).

a total molecular weight of only 6 millions like the poliomyelitis virus or of 300 millions like the influenza virus. It should be noted, furthermore, that ribonucleoprotein particles with a similar amount of nucleic acid have also been found in the microsomes of uninfected cells (CHENG[13]). This nucleic acid content of 2×10^6, although present in many viruses, therefore does not seem to be a unique property of viruses.

The X-ray crystallographic studies of FRANKLIN, KLUG *et al.* (FRANKLIN[14], FRANKLIN and HOLMES[15], FRANKLIN and KLUG[16]) have made it possible to design a model showing the way in which the different constituents of the virus are linked together (Fig. 2). From this it appears that the nucleic acid lies like a helical coil inside the virus with an empty space in the center, whereas the protein subunits form a cover around the nucleic acid.

Electron micrographs of tobacco mosaic virus treated in several ways seem to confirm this structure. The existence of the hollow space inside has been proved by the fact that it can be filled with a contrast medium (HUXLEY[17]). Figure 1 shows a virus preparation treated this way where every virus particle shows a dark area in the center due to the presence of the contrast medium.

The protein can also be removed by various chemical treatments such as weak alkaline solution (SCHRAMM *et al.*[18]) or sodium dodecyl sulfate (HART[19]). If this treatment is carried out very briefly the protein is only partially removed and various intact parts of the virus appear to be linked only by a thin thread in the center which corresponds to the nucleic acid (Fig. 3). In the absence of the protein coat, the virus loses its rigidity and becomes flexible at the places where the protein has been removed (Fig. 4).

NIXON and WOODS[20], furthermore, were able to demonstrate by electron microscopy the helical structure of the protein subunits as portrayed by FRANKLIN *et al.* by their x-ray studies.

[13] P. Y. CHENG, Biochim. biophys. Acta *37*, 238 (1960).
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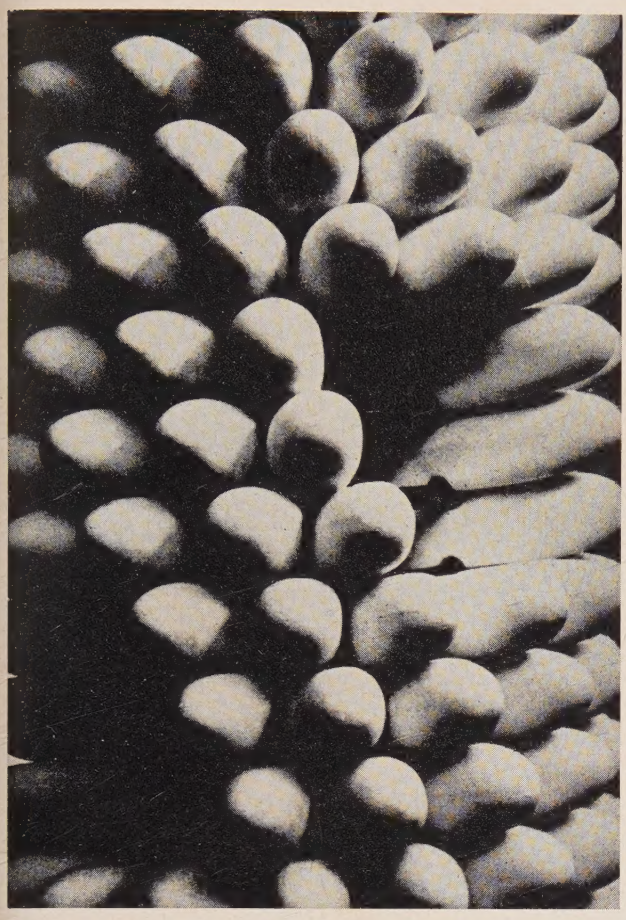

Fig. 2. Model of tobacco mosaic virus (FRANKLIN *et al.*[21] 1956).

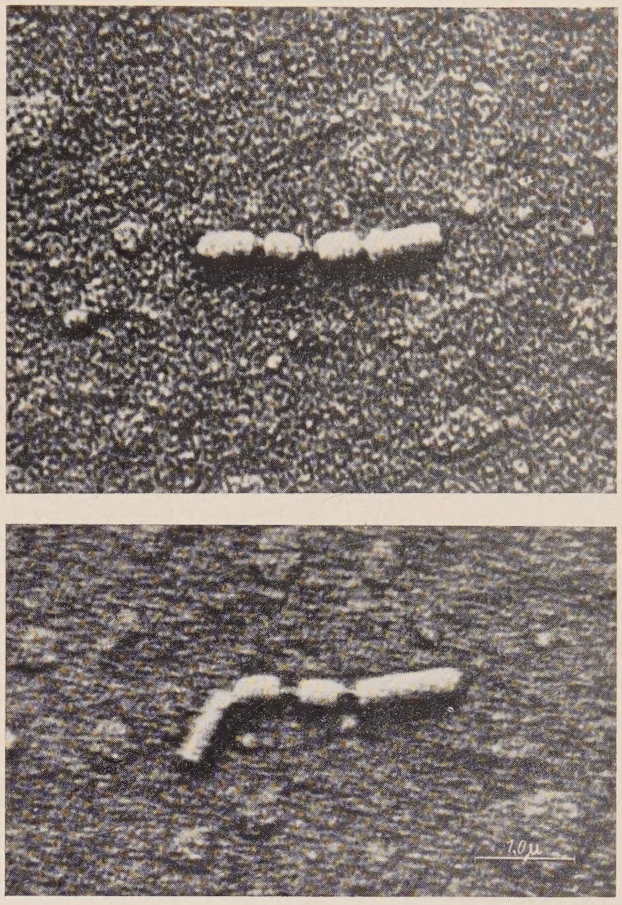

Fig. 3 and 4. Tobacco mosaic virus after partial removal of its protein at pH 10·3. (Courtesy of Prof. G. SCHRAMM, Tübingen.

Pathological changes caused by TMV. The tobacco mosaic virus causes two kinds of pathological changes according to the nature of the host. Some species of plants, such as *Nicotiana glutinosa*, react by the formation of a number of local lesions on each infected leaf. These local lesions appear after a few days and expand only very slowly (Fig. 5). The number of lesions is dependent on the concentration of the virus in the solution which has been rubbed on the leaf. The infectivity of a virus preparation or a virus nucleic acid can, therefore, be measured by the number of necroses which appear on a number of half leaves after infection as compared to the number of necroses caused by a control solution of known virus concentration.

In contrast, in some other varieties of tobacco plants, such as the 'Turkish tobacco', *Nicotiana tabacum*, the infection spreads rapidly over the whole plant causing a systemic infection. The leaves show diffuse discolorations rather than local lesions (Fig. 6). These so-called systemic hosts are preferentially used for the preparation of large quantities of virus. They can also be used to study the time-course of virus production by measuring the virus present in identical leaf disks at various time intervals.

Infectivity of tobacco mosaic virus nucleic acid. It was found by GIERER and SCHRAMM[7], as well as by FRAENKEL-CONRAT[8], that the application of the isolated ribonucleic acid on leaves of local lesion hosts does also result in the formation of local necroses and causes the formation of new virus. Not only the intact virus but also its isolated nucleic acid are therefore capable of initiating a viral infection.

Since then, the infectivity of many other virus nucleic acids has been described (COLTER[9]). Even though the preparations of many animal viruses were not quite pure, the residual activity after phenol extraction was sensitive to ribonuclease which indicated that it was due to the free virus nucleic acid. Infectious nucleic acid preparations have also been isolated from three other purified plant viruses (KAPER and STEERE[22], RUSHIZKY and KNIGHT[23]).

The infectivity of the isolated nucleic acid from tobacco mosaic virus is quite appreciably lower than the

[21] R. E. FRANKLIN, D. L. D. CASPAR, and A. KLUG, *Plant Pathology-Problems and Progress*, 1908–1958 (Academic Press, New York). – CASPAR and KLUG, Advanc. Virus Res. *7* (in press).

[22] J. M. KAPER and R. L. STEERE, Virology *7*, 127 (1958), *8*, 527 (1959).

[23] G. W. RUSHIZKY and C. A. KNIGHT, Virology *8*, 448 (1959).

infectivity of the intact tobacco mosaic virus. This appears especially if the comparison is not carried out on a weight basis, but if identical amounts of free nucleic acid and of nucleic acid in the intact virus are compared. The activity of free nucleic acid compared with that of an intact virus containing the same amount of ribonucleic acid ranges from 0·1–2%. This lower infectivity does not appear to represent a lower intrinsic capacity of the nucleic acid to cause an infection but is rather due to the fact that it is much more accessible to destruction by cellular enzymes than the nucleic acid inside the virus which is protected by its protein coat.

Highly purified infectious nucleic acid preparations have a protein content of less than 0·2% (Ramachandran and Fraenkel-Conrat[24]). The infectivity therefore seems to lie entirely in the nucleic acid. Whether there is a small peptide attached to the nucleic acid which might be of some importance, cannot, however, be excluded with certainty. An interesting biological difference in the infectivity of the free nucleic acid and the intact virus has been described by Schramm and Engler[25] and by Fraenkel-Conrat *et al.*[26]. After infection with the free nucleic acid, there is a shorter latent period until new virus is produced and until the necroses appear. The free nucleic acid, therefore, seems to initiate an infection faster than the intact virus.

Splitting the virus. The protein and the nucleic acid of the virus can be separated in various ways:

a) Treatment with sodium dodecyl sulfate: By adding about 1% sodium dodecyl sulfate to a virus solution at a slightly alkaline or acid pH the two components separate. By adding ammonium sulfate to a concentration of 33% the protein is precipitated already at room temperature and can be removed by centrifugation, whereas the nucleic acid precipitates only later upon standing in the cold (Fraenkel-Conrat *et al.*[27]).

b) Phenol extraction: If phenol saturated with water is added to a virus solution with rapid stirring an emulsion is formed. Upon centrifugation the protein is dissolved in the phenol layer or settles as a layer between the two phases, whereas the nucleic acid remains in the water phase (Schuster, Schramm and Zillig[28]).

c) Upon short heating of a virus in a solution of salts, the protein denatures and can be removed by centrifugation, whereas the nucleic acid remains in solution (Cohen and Stanley[29], Knight[30]).

d) Acetic acid: If two volumes of glacial acetic acid are added to a virus solution, in contrast to the previous methods, the protein stays in solution and the nucleic acid is precipitated (Fraenkel-Conrat[31]).

Only the first two methods have provided infectious nucleic acids from tobacco mosaic virus. Heating has been successful in the preparation of an infectious ribonucleic acid from other plant viruses (Kaper and Steere[22]). The last method does not give an infectious nucleic acid but the protein seems to be particularly well preserved and native.

Chemical modifications of the infectivity of tobacco mosaic virus nucleic acid. Chemical alterations in the virus nucleic acid can be induced in two ways, either by mo-

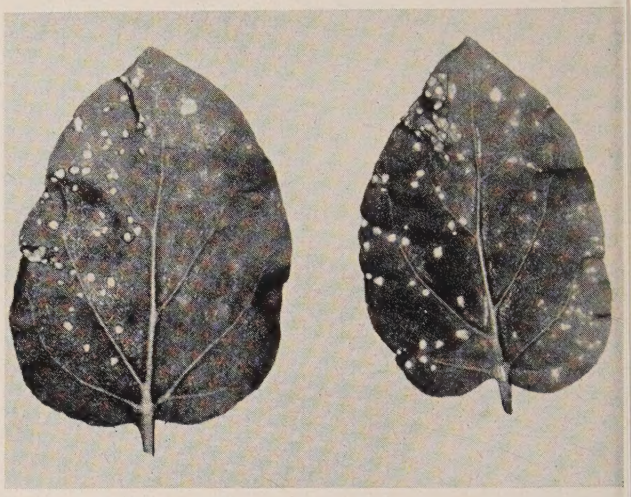

Fig. 5. Tobacco leaves infected with TMV, local lesion host (*Nicotiana glutinosa*). Courtesy of Dr. K. M. Smith, Cambridge.

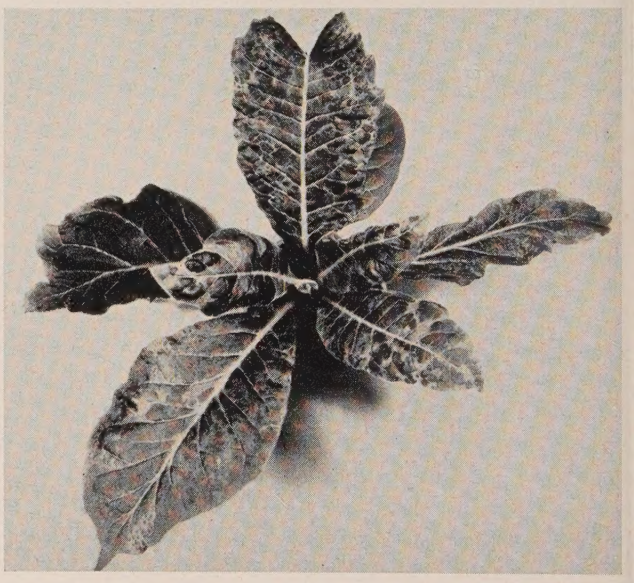

Fig. 6. Tobacco plant infected with TMV, systemic host (*Nicotiana tabacum*). Courtesy of Dr. K. M. Smith, Cambridge.

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[27] H. Fraenkel-Conrat, B. Singer, and R. C. Williams, Biochim. biophys. Acta *25*, 87 (1957).
[28] H. Schuster, G. Schramm, and W. Zillig, Z. Naturf. *11b*, 339 (1956).
[29] S. S. Cohen and W. M. Stanley, J. biol. Chem. *144*, 589 (1942).
[30] C. A. Knight, J. biol. Chem. *197*, 241 (1952).
[31] H. Fraenkel-Conrat, Virology *4*, 1 (1959).

difying the nucleic acid acid after it has been formed or by incorporating an unnatural base into the nucleic acid while the virus is growing. The first way is the general mechanism for the inactivation of a virus, whereas the second is usually encountered upon the addition of structural analogs to infected plants. In the following text, examples will be presented for both these mechanisms of chemical modifications which change the infectivity of tobacco mosaic virus.

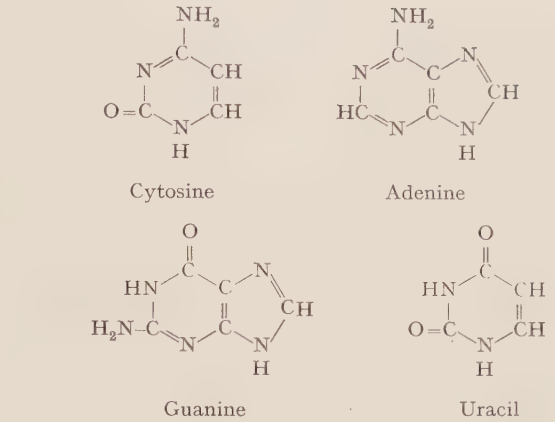
Reaction with formaldehyde. One of the chemicals used most widely for the inactivation of viruses is formaldehyde. It has found extensive application in the preparation of various vaccines, e.g. the Salk vaccine against poliomyelitis virus. Formaldehyde is a very reactive substance with a particular tendency towards polymerization. It has also been widely used in the tanning of skins, and the chemical reactions between formaldehyde and proteins have been extensively studied (French and Edsall[32]). Especially the studies of Fraenkel-Conrat *et al.*[33]) have provided evidence for a two step reaction. In a first step formaldehyde reacts with an amino group in the protein molecule to form a methylol derivative (Reaction I). In a second step the hydroxyl group of this methylol derivative reacts with another amino group or any active hydrogen atom and thus forms a methylene bridge (Reaction II).

$$\text{I}\quad R\text{–}NH_2 + HCHO \rightleftharpoons R\text{–}NH\text{–}CH_2OH$$

$$\text{II}\quad R\text{–}NH\text{–}CH_2OH + H_2N\text{–}R \longrightarrow R\text{–}NH\text{–}CH_2\text{–}NH\text{–}R$$

Reactions of this kind are certainly encountered when formaldehyde is allowed to act upon a virus. It is doubtful, however, whether it is the reaction with the virus protein which leads to inactivation, since there are a number of very active protein reagents which, although they react with the virus, do not cause inactivation. It seems not unlikely, therefore, that the principal action of formaldehyde might be its chemical reactivity towards the nucleic acid rather than towards the protein.

Fraenkel-Conrat[34] has demonstrated that formaldehyde causes very marked changes in the ultraviolet spectrum of ribonucleic acids. Using formaldehyde labelled with C^{14}, it has been possible to study quantitatively the reaction of formaldehyde with ribonucleic acid (Staehelin[35]). Figure 7 shows schematically the linking of a few nucleotides as it occurs in ribonucleic acids in which, however, many hundreds of nucleotides are linked in this manner. The data of Gierer[10] and Boedtker[11] actually suggest that the ribonucleic acid of tobacco mosaic virus has a molecular weight of about 2×10^6 and consists of about 6000 nucleotides linked together in one chain. The only groups capable of reacting with formaldehyde are the amino groups of the bases, i.e. of adenine, guanine and cytosine since uracil does not carry an amino group.



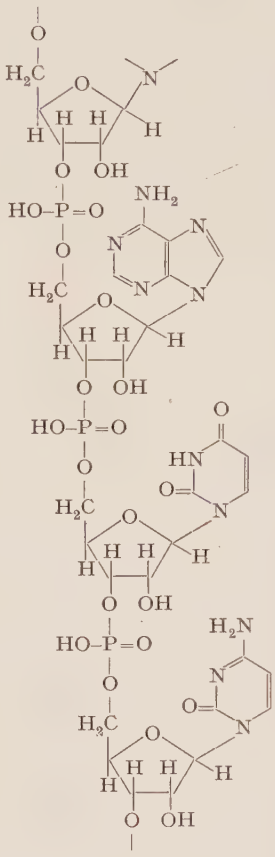


Fig. 7. Linking of nucleotides in ribonucleic acids.

This was demonstrated by the use of synthetic polynucleotides which are formed by the enzyme polynucleotide phosphorylase (Grunberg-Manago *et al.*[36]) and which contain only one base. Whereas polynucleotides containing adenine and cytosine (Poly A and Poly C) react very readily with formaldehyde, polynucleotides containing only uracil (Poly U) do not react at all (Staehelin[35]).

Intact ribonucleic acids also react accordingly. In order to express the extent of the reaction quantitatively, the binding of formaldehyde to the ribonucleic acid has been related to the number of nucleotides. It can be seen in Figure 8 that the reaction stops when about 5·5–6% formaldehyde have been bound to the nucleic acid on a weight basis. Since the molecular weight of formaldehyde is less than one tenth of that of an average nucleotide, it can be said on a molar basis that somewhere between 60 and 70 molecules of formaldehyde have reacted with about 100 nucleotides. According to the base composition of the tobacco mosaic virus nucleic acid, the bases which carry an amino group, i.e. adenine, guanine and cytosine, correspond

[32] D. French and J. T. Edsall, Advanc. Protein Chem. *2*, 278 (1945).

[33] H. Fraenkel-Conrat and H. S. Olcott, J. Amer. chem. Soc. *70*, 2673 (1948).

[34] H. Fraenkel-Conrat, Biochim. biophys. Acta *15*, 307 (1954).

[35] M. Staehelin, Biochim. biophys. Acta *29*, 410 (1958).

[36] M. Grunberg-Manago, P. J. Ortiz, and S. Ochoa, Biochim. biophys. Acta *20*, 269 (1956).

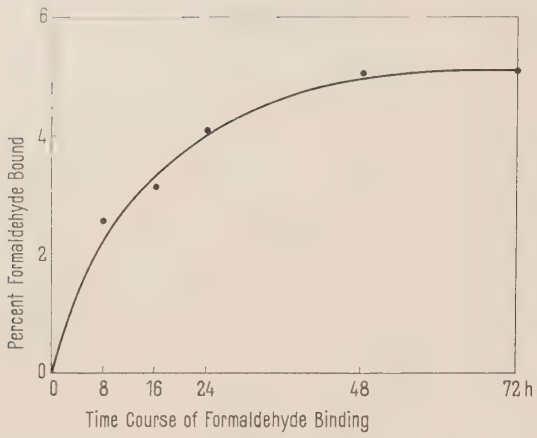


Fig. 8. Reaction of formaldehyde with nucleic acid from tobacco mosaic virus. The reaction was carried out according to Staehelin[35] (1958) with formaldehyde C^{14} and the amount of formaldehyde bound expressed in mg of formaldehyde/100 mg of nucleic acid.

Tab. I. Inactivation of Nucleic Acid[a] (Staehelin[41])

Inactivating agent	Concentration %	Activity %
Formaldehyde	0·1	0
	0·05	4
	0·025	34
Glyoxal	0·1	6
	0·05	23
	0·025	81
	0·0125	100
Kethoxal	0·02	1
	0·01	8
	0·005	32
	0·0025	73

[a] TMV-RNA (1 mg/ml) was incubated as in Table I for 30 min at 23°. After the reaction the nucleic acid was precipitated 6 times with alcohol to remove all free inhibitor and then assayed.

Tab. II. Inactivation of Intact TMV[a] (Staehelin[41])

Inactivating agent	Concentration %	Temperature	Time h	Activity % of original
Formaldehyde	1	23°	8	41
			16	33
			24	9
Glyoxal	2	23°	8	52
			16	19
			24	11
Kethoxal	2	23°	8	60
(β-ethoxy-α-keto-butyraldehyde)			16	63
			24	57
	4	23°	8	81
			16	53
			24	21
	4	37°	8	31
			16	29
			24	20

[a] TMV (1 mg/ml) was incubated at room temperature in 0·001 M phosphate buffer pH 6·8 with the concentrations of inhibitors and for the times indicated. After the reaction the solutions were dialyzed overnight against the same buffer in the cold and assayed.

to about 71% of the total bases. The binding of 60–70 molecules of formaldehyde per 100 nucleotides indicates that almost every amino group has reacted with one molecule of formaldehyde, probably in a manner very similar to the mechanism described for proteins (Reactions I and II).

In the presence of higher salt concentrations, or already of low concentrations of divalent kations, the reaction is less extensive because a great number of the amino groups have become unreactive, probably through the formation of hydrogen bonds between the bases (Staehelin[37]).

The reaction proceeds very slowly at low formaldehyde concentrations (Fig. 8). Inactivation of the nucleic acid, however, occurs very fast and the infectivity of the free nucleic acid is lost when only a few molecules of formaldehyde have reacted with the many thousand amino groups present in the nucleic acid (Table I). This great sensitivity of the isolated ribonucleic acid is in contrast to the relative resistance of the intact virus towards formaldehyde treatment. Tobacco mosaic virus is inactivated only at about 100 times greater concentrations of formaldehyde and after longer times of treatment (Table II). This seems rather surprising since the virus contains the nucleic acid as its active component. One has to assume, therefore, that the amino groups of the ribonucleic acid are not accessible in the intact virus. This inaccessibility could be brought about in two ways:

a) The amino groups could be involved in strong hydrogen bonding in the intact virus, as in deoxyribonucleic acid, which also does not react readily with formaldehyde, or

b) The presence of the protein coat surrounding the nucleic acid could prevent access of the formaldehyde to the nucleic acid.

There is good evidence in favour of the second mechanism, and, especially by comparing the effect of various inactivating chemicals with similar properties but of different size, it was possible to show the importance of the problem of penetration into the virus. This will be shown in the following experiments.

Reaction with glyoxal derivatives. Glyoxal derivatives have been shown by various investigators to inactivate viruses *in vitro* (De Bock *et al.*[38], McLimans *et al.*[39]). A few have also had a chemoprophylactic effect on virus infections in eggs. Lately some glyoxal derivatives have been reported to have chemoprophylactic activity in animals (Cavallini *et al.*[40]).

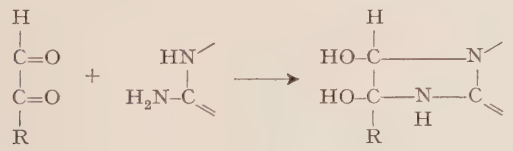
[37] M. Staehelin, Exper. *15*, 413 (1959 a).

[38] C. A. De Bock, J. Brug, and J. N. Walop, Nature, Lond. *179*, 706 (1957).

[39] W. F. McLimans, G. E. Underwood, E. A. Slater, E. V. Davis, and R. A. Siems, J. Immunol. *78*, 104 (1957).

[40] G. Cavallini and E. Massarani, J. med. pharm. Chem. *1*, 365 (1959). – G. Cavallini, E. Massarani, D. Nardi, F. Magrassi, P. Altucci, G. Lorenzutti, and U. Sapio, J. med. pharm. Chem. *1*, 601 (1959).

Glyoxal derivatives react with nucleic acids in a way quite different from formaldehyde. In contrast to the marked shift of the maximum to higher wave lenghts and the general increase in the height of the absorption maximum caused by formaldehyde, there is only a very slight shift of the maximum in the opposite direction, i. e. towards shorter wave lengths, and no increase in the absorption upon treatment with glyoxal derivatives (STAEHELIN[41]). Formaldehyde causes changes in the ultraviolet spectrum similar to those with nucleic acids also with free nucleotides, i.e. adenylic, guanylic, and cytidylic acids, but glyoxal derivatives only change the spectrum of one nucleotide, namely guanylic acid. By using a radioactive glyoxal derivative, it can also be shown that only guanylic acid out of all four nucleotides reacts. Figure 9 shows the electrophoretic separation of a mixture of four nucleotides after it has reacted with a radiaoctive glyoxal derivative (Kethoxal). From the radio-autogram to the left, it can be seen that only guanylic acid has become labelled but that the reaction with this nucleotide has been complete, since after the reaction the entire guanylic acid band, as seen in the ultraviolet light, migrates with a velocity corresponding to the reaction product with the glyoxal derivative rather than to free guanylic acid. Since glyoxal derivatives react with all compounds containing the diamine structure shown below, it appears most likely that they react with guanine in the following manner under the formation of a stable 5-membered ring (Reaction III).



III

This reaction takes place much more readily and much faster than the formaldehyde reaction. Accordingly, the inactivation of the isolated ribonucleic acid from tobacco mosaic virus occurs much faster, especially by stable glyoxal derivatives such as Kethoxal (β-ethoxy-α-ketobutyraldehyde), than by formaldehyde. Glyoxal itself is less active because of its marked instability (Tab. II).

Interestingly, towards the intact virus Kethoxal is much less active than glyoxal, whereas formaldehyde is the most active compound of all (Table I). The order of activity towards the intact virus, therefore, follows the size of the compounds, the activity being greater the smaller the compound. This is probably most noteworthy in the case of glyoxal and Kethoxal, which have the same mechanism of action but whose order of activity is reversed according to whether they are assayed on the free nucleic acid or on the intact virus.

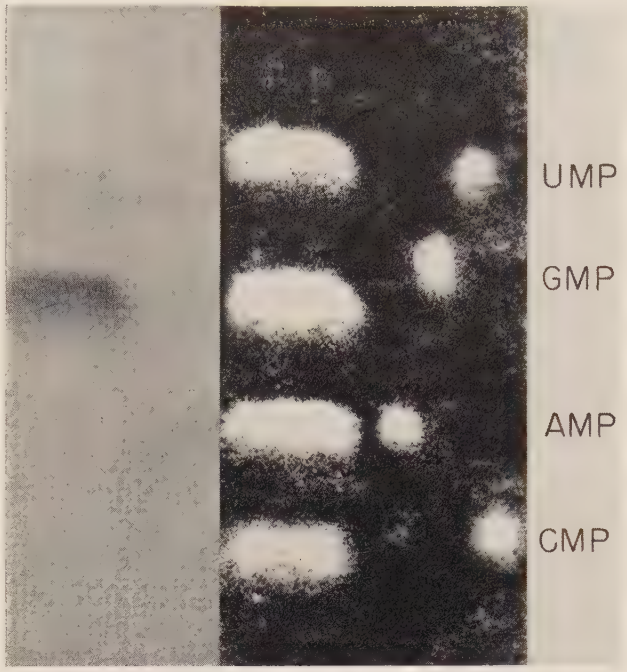


Fig. 9. Reaction of nucleotides with Kethoxal-C^{14}. Adenylic, guanylic, cytidylic and uridylic acids (all 0·005 M) were incubated in 0·01 M phosphate buffer pH 7 with 1% Kethoxal-C^{14} for 16 h at room temperature. The mixture was then separated by paper electrophoresis at pH 3·5 in 0·05 M ammonium formate. The ultraviolet absorption of the four separated bands is shown on the right hand side together with the four markers. The radioautogram of the same experiment is shown on the left hand side.

It appears that towards the free nucleic acid Kethoxal is more active than glyoxal because of its greater stability, but that its larger size might render it less active towards the intact virus because it prevents it from penetrating easily towards the nucleic acid in the center of the virus. The finding that the very small molecule formaldehyde, although being less active towards the free nucleic acid, is the most active compound towards the intact virus, also favours the concept that the ability of a compound to penetrate to the nucleic acid through the protein coat determines to a great extent its power to inactivate a virus. It might be noted that the much higher concentrations of inactivating compounds needed to inactivate the intact virus also indicates that the nucleic acid is highly protected in the intact virus but that these very high concentrations might ultimately result in its chemical inactivation.

Reconstitution of an inactive virus. The inactivating agents mentioned so far react with proteins as well as with nucleic acids. It is difficult, therefore, to show the effect on the nucleic acid in the intact virus separately. But it has been possible to prepare a virus in which only the nucleic acid moiety has been modified chemically.

[41] M. STAEHELIN, Biochim. biophys. Acta *31*, 448 (1959 b).

For this purpose, use was made of the ability of the two constituents of TMV. i.e. the protein and the nucleic acid, to reconstitute again and form an intact virus particle. Fraenkel-Conrat and Williams[42] have first made the extremely interesting observation that upon combining a solution of pure nucleic acid with a solution of pure protein, under suitable conditions, intact virus particles are formed again from the two constituents. This reconstitution can proceed to the extent of 30–80% of the material present (Fraenkel-Conrat *et al.*[43]). The reconstituted virus is fully infective and in the electron microscope appears to have the same structure as the original tobacco mosaic virus. The splitting and the reconstitution of the virus are shown schematically in Figure 10. The same drawing also shows the processes involved in the production of reconstituted virus from inactivated nucleic acid. In this experiment the virus is first split and the nucleic acid isolated. This nucleic acid is then inactivated by some chemical treatment and recombined with untreated protein to form intact virus again.

Table III shows the results of such an experiment using formaldehyde as an inactivating agent of the nucleic acid. It can be seen that the reconstitution proceeds to a similar extent with the inactivated nucleic acid as with untreated nucleic acid, and that the infectivity of the reconstituted virus closely parallels the infectivity of the nucleic acid used for reconstitution. In addition to formaldehyde, a number of different inactivating agents can be used (Fraenkel-Conrat, Staehelin and Crawford[44]), e.g. epoxydes, β-propiolactone, HNO_2, ultraviolet light, etc. After treatment with all these agents, the nucleic acid has still the ability to reconstitute intact but inactive virus. The only requirement appears to be the intactness of the entire chain length of the nucleic acid strand. If the nucleic acid is inactivated by ribonuclease, for instance, the ability to reconstitute rapidly diminishes.

The characteristics of this inactive reconstituted virus are shown in Figure 11. Its special advantage over the formaldehyde inactivated virus is its unaltered antigenic property. Whereas in the intact virus the protein also reacts extensively with formaldehyde which alters its antigenic structure, in the reconstituted virus the inactivated nucleic acid is surrounded by a native protein. This makes it an antigen which resembles the untreated virus much more closely than does the formaldehyde treated virus. This seems to be confirmed by its serological reactions. Cross reactions of sera obtained by injecting rabbits with native TMV and with inactive reconstituted TMV have indicated that the two viruses behave as identical antigens. Formaldehyde inactivated TMV, by contrast, was precipitated only at a somewhat higher titer by normal ANTI-TMV serum than intact TMV and inactive reconstituted TMV.

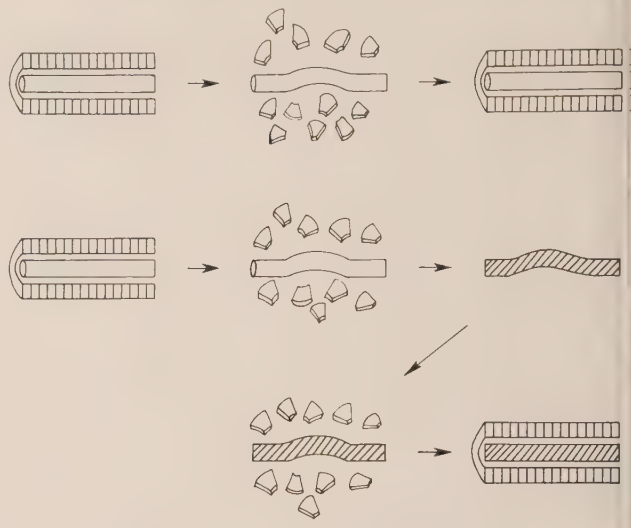


Fig. 10. Splitting of tobacco mosaic virus and reconstitution of active (top) and inactive virus (bottom).

Tab. III. Reconstitution of formaldehyde inactivated nucleic acid with native protein

Time of inactivation	Activity of nucleic acid % of controls	Activity of reconstituted virus % of controls	Yield of reconstitution % of theory
0	100	100	33
1	81	85	27
3	6	7	30
6	2	3	28

(from Staehelin[45])

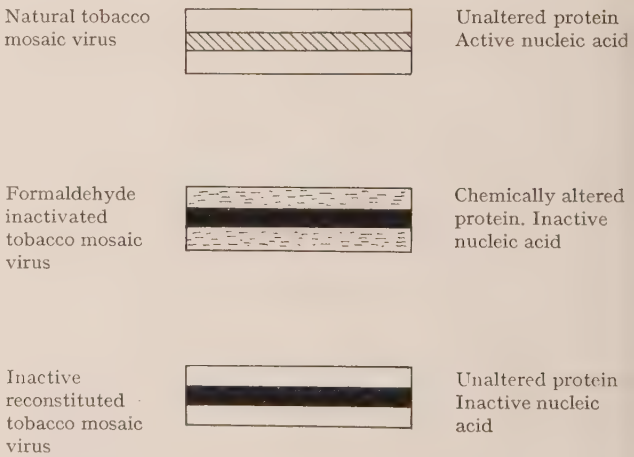


Fig. 11. Characteristics of natural TMV, formaldehyde inactivated TMV and inactive reconstituted TMV.

[42] H. Fraenkel-Conrat and R. C. Williams, Proc. nat. Acad. Sci. Wash. *41*, 690 (1955).

[43] H. Fraenkel-Conrat and B. Singer, Biochim. biophys. Acta *33*, 359 (1959).

[44] H. Fraenkel-Conrat, M. Staehelin, and L. Crawford, Proc. Soc. exp. Biol. *102*, 118 (1959).

[45] M. Staehelin, Helv. physiol. Acta *17*, [C] 40 (1959 c).

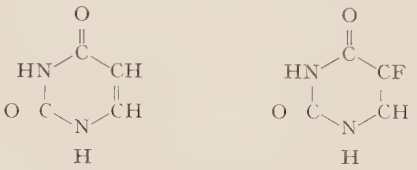
Reaction with nitrous acid. Schuster and Schramm[46] have studied the effect of nitrous acid on the biological properties of tobacco mosaic virus nucleic acid. Nitrous acid causes a deamination of the purine and pyrimidine bases to their respective hydroxyl derivatives. Thus, cytosine is converted into uracil, guanine into xanthine and adenine into hypoxanthine. From the kinetics of the reaction, they concluded that the deamination of one of 3300 nucleotides of the nucleic acid results in an inactivation of the molecule.

Gierer and Mundry[47] made the extremely interesting observation that, upon progressing inactivation, the relative proportion of mutants steadily increased. After an inactivation of over 95% of the molecules, more than 50% of the survivors turned out to be mutants. This finding is unique for the inactivation with nitrous acid and can possibly be correlated with the fact that one of the three reactions taking place, i.e. the conversion of cytosine to uracil does not result in the formation of an unnatural derivative but in a transformation of one natural base into another. The kinetics of the reaction indicate that one single transformation is sufficient to cause a mutation. It would appear, therefore, that the mechanism of mutation consists in the alteration of only one of the 6000 nucleotides into another natural nucleotide.

Incorporation of purine and pyrimidine analogs. A chemical modification of the virus infectivity can also be obtained by incorporating structural analogs into the nucleic acid of viruses. In this case the virus is not altered after it is formed but is already produced in the cell as an unnatural modification. The incorporation of structural analogs has been studied quite extensively in tobacco mosaic virus. At least three unnatural bases, i.e. 8-azaguanine (Matthews and Smith[48]), 2-thiouracil (Jeener[49]) and 5-fluorouracil (Gordon and Staehelin[50]), have been shown to be incorporated into tobacco mosaic virus. In all three instances the virus containing the analog differed in its biological characteristics from normal TMV.

One of the simplest ways of incorporating structural analogs into TMV is to infect leaves of a systemic host plant and to let them float on a solution of the structural analog. Virus growth proceeds readily also in detached leaves, especially under strong illumination. This method can also be used quantitatively to determine the effect of a structural analog on the growth of virus by excising identical disks from a large number of leaves (e.g. with a cork borer). The amount of virus produced in a certain number of leaf disks in the presence of various concentrations of the analog can then be determined spectrophotometrically after some preliminary steps of purification. The effect of the analog is expressed by comparing the virus yield in the leaf disks grown in a solution of the analog with those grown in water.

5-Fluorouracil. 5-Fluorouracil being a close analog of uracil has been examined in this manner. It was found to inhibit the growth of TMV in leaf disks, although rather high concentrations of the analog were required (Gordon and Staehelin[50]).



Uracil 5-Fluorouracil

The riboside, 5-fluorouridine, was somewhat more active than the free base. It was found, furthermore, that the extent to which the virus growth was inhibited was not only a function of the nature and the concentration of the analog but also of the time at which the analog was added. The inhibition was more pronounced the sooner after the infection the analog was added (Fig. 12), although there was only very little virus produced during the first few days.

The inhibition, even at very high concentrations of 5-fluorouracil, was never complete and it was of special interest to study the virus grown in the presence of the analog. This virus was found to contain 5-fluorouracil to a rather high extent (Gordon and Staehelin[50]). The analog replaced the normal base uracil since the sum of 5-fluorouracil and uracil always corresponded to the uracil content of normal TMV. The presence of 5-fluorouracil during the growth of the virus, however, resulted in a replacement of 30–50% of the uracil by the the structural analog. The nucleic acid of this virus,

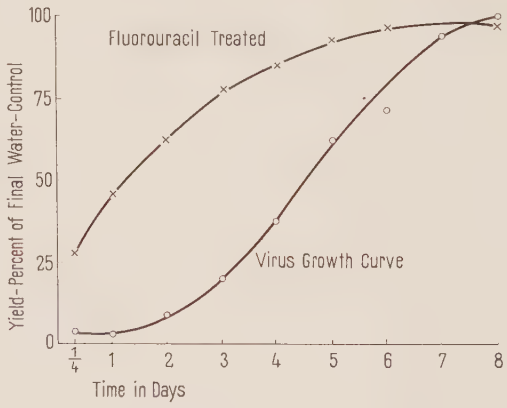


Fig. 12. Inhibition of the growth of TMV by the addition of 5-fluorouracil at various times (from Gordon and Staehelin[50]).
○ virus growth curve. × virus yield on the eighth day after addition of 5-fluorouracil at various times in percent of the virus yield in the absence of 5-fluorouracil.

[46] H. Schuster and G. Schramm, Z. Naturf. *13b*, 697 (1958).
[47] A. Gierer and K. W. Mundry, Nature, Lond. *182*, 1457 (1958).
[48] R. F. C. Matthews and J. D. Smith, Adv. Virus Res. *3*, 49 (1955).
[49] R. Jeener, Biochim. biophys. Acta *23*, 351 (1957).
[50] M. P. Gordon and M, Staehelin, Biochim. biophys. Acta *36*, 351 (1959).

therefore, contains in its nucleic acid about 500–800 molecules of 5-fluorouracil. It would be expected that this high extent of replacement of a natural base by an unnatural analog would result in some profound changes of its biological properties.

Infectivity of TMV containing 5-fluorouracil. The infectivity of a tobacco mosaic virus can be tested in either of two ways. When applied to leaves of a local lesion host (Fig. 5), the number of local necroses is proportional to the number of infective particles present in the solution. On local lesion hosts, the infectivity of the virus containing 5-fluorouracil appeared to be identical with that of normal TMV, i.e. solutions of both viruses in equal concentrations caused the same number of local lesions on the leaves. This test, however, does not give any information about the rate at which the virus is formed unless the time of the appearance of the lesions is very accurately measured. In a more accurate way, the rate of virus growth can be followed by infecting leaves of a systemic host (Fig. 6). Opposite half leaves were infected with normal and with 5-fluorouracil containing virus. Disks of identical size were then prepared and floated on water, and the amount of virus produced in a certain number of disks was determined at different times. When assayed this way on a systemic host, the virus containing 5-fluorouracil was definitely less infective than ordinary TMV (Fig. 13). These apparently conflicting results can be summarized as follows:

a) The virus containing the analog has the same number of infective virus particles as the natural virus since it causes the same number of necroses on local lesion hosts.

b) After infection with the unnatural virus, growth of new virus proceeds less rapidly since the virus yield at given times after the infection in systemic hosts is smaller than after infection with normal TMV.

These findings can be explained if we depict the processes which lead to virus infection in the following manner (Fig. 14). From analogy with phages, we assume that at one point on the surface or inside the cell nucleic acid is set free from the virus. This nucleic acid then transfers its information to the cell and provokes a series of highly specific processes in the cell, about which very little is known at present but which finally lead to the production of new virus. It would be expected that the presence of an unnatural base in the nucleic acid would exert its effect at the time when the nucleic acid reacts first with the cell. If the unnatural base very closely resembles the natural base, it can well be imagined that the nucleic acid is not inactive but the time required to give the information to the cell is prolonged. This would cause a prolongation of the latent period, i.e. the time before the onset of production of new virus (Fig. 14). This assumption is supported by the finding that the incorporation of 5-fluorouracil causes a quantitative but never a qualitative change in the virus infection. In spite of a very thorough study, we were never able to detect the formation of mutants after infection with unnatural virus, although GIERER and MUNDRY[47] have shown that the alteration of one single base into another natural base might result in the production of a new mutant. This would indicate that the cell finally recognizes the 5-fluorouracil molecule as uracil.

This retardation of the onset of a new infection caused by the incorporation of 5-fluorouracil into the virus might also explain the strong time dependence of the inhibiting effect of 5-fluorouracil on the growth of TMV. This time dependence is most marked at the very early stages after infection, i.e. in the latent period

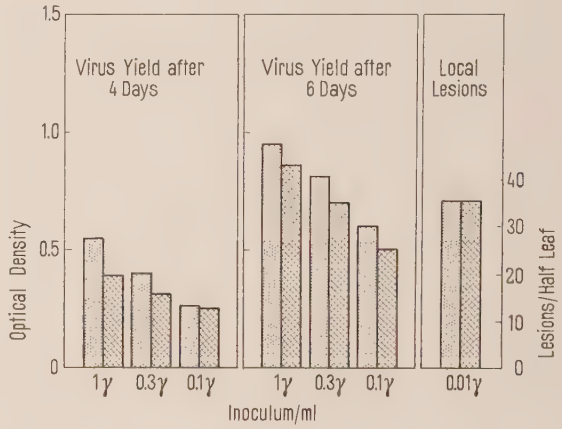


Fig. 13. Infectivity of TMV containing 5-fluorouracil. Left columns: normal TMV. Right columns: 5-Fluorouracil containing TMV.

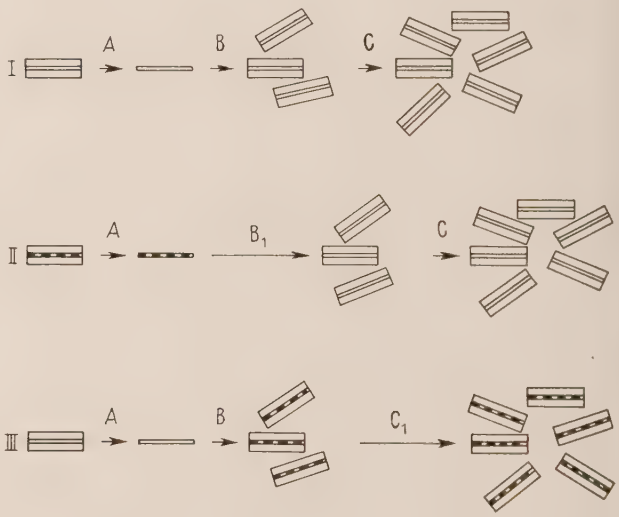


Fig. 14. Effect of 5-fluorouracil incorporation on the processes leading to virus production.
A) Liberation of nucleic acid. B) Transfer of information from nucleic acid to infected cell and formation of first new virus particles. C) Spreading of virus infection by first newly formed virus particles. I. Normal virus infection. II. Infection with virus containing 5-fluorouracil. Steb B delayed. III. Infection in the presence of 5-fluorouracil (incorporation of 5-fluorouracil into the first newly formed virus particles resulting in a delayed further spreading of the infection). Step C delayed.

long before the bulk of virus is formed. If 5-fluorouracil is so rapidly incorporated into TMV, it would be expected that upon very early addition of 5-fluorouracil already the earliest new forms of virus contain the analog. But it is these very first new viruses, possibly even nucleic acids only, which are responsible for the spreading of the infection from cell to cell. Since the the incorporation of 5-fluorouracil results in a slower rate of virus growth, the spreading of the infection by these early stages would be inhibited. This would result in a reduction of the virus yield which is in accord with the experimental findings (Fig. 14),

This delayed spreading of the virus infection does not explain the inhibition of the virus growth by 5-fluorouracil entirely, however, since 5-fluorouracil in addition causes an inhibition of the synthesis of ribonucleic acid in general (STAEHELIN and GORDON[51]). But it might be responsible for the very marked time dependence of the addition of 5-fluorouracil very soon after the infection.

Zusammenfassung

Die biologischen Eigenschaften des Tabakmosaikvirus können durch chemische Umsetzungen seiner Nukleinsäure in verschiedener Weise beeinflusst werden:

1. Formaldehyd reagiert mit den Aminogruppen der Basen, Glyoxal und seine Derivate speziell mit der Diaminstruktur des Guanins. Beide Reaktionen bewirken eine Inaktivierung des Virus.

2. Durch Behandlung der isolierten Nukleinsäure und nachherige Rekonstitution mit nativem Eiweiss konnte ein Virus gewonnen werden, dessen antigene Eigenschaften denjenigen des unbehandelten Virus entsprechen, das jedoch inaktiv ist.

3. Reaktion mit salpetriger Säure, bei der unter anderem Cytosin in Uracil übergeführt wird, führt ausser zu Inaktivierung auch zu einer grossen Anzahl von Mutanten.

4. Der Einbau strukturanaloger Basen, wie z. B. des 5-Fluoruracils, führt zu einem Virus, das zwar eine Infektion hervorrufen kann, bei der jedoch die Wachstumsgeschwindigkeit des neuen Virus verzögert ist.

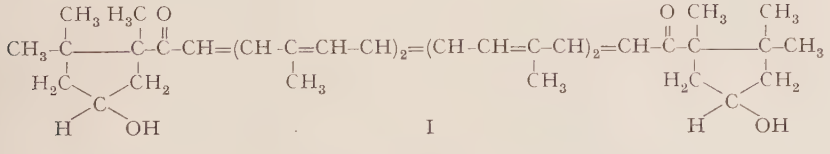
[51] M. STAEHELIN and M. P. GORDON, Biochim. biophys. Acta *38*, 307 (1960).

Brèves communications - Kurze Mitteilungen
Brevi comunicazioni - Brief Reports

Les auteurs sont seuls responsables des opinions exprimées dans ces communications. – Für die kurzen Mitteilungen ist ausschliesslich der Autor verantwortlich. – Per le brevi communicazioni è responsabile solo l'autore. – The editors do not hold themselves responsible for the opinions expressed by their correspondents.

Bemerkungen zur Struktur des Capsorubins

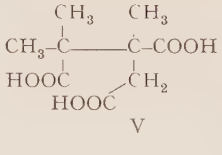
Es wurde bereits berichtet, dass die richtige Bruttoformel des Capsanthins $C_{40}H_{56}O_3$ und die des Capsorubins $C_{40}H_{56}O_4$ beträgt[1]. Vor kurzem haben ENTSCHEL und KARRER[2] sowie BARBER, JACKMAN, WARREN und WEEDON[3] die Struktur des Capsanthins und des Capsorubins (I) festgestellt, doch konnte die Lage der Substituenten am Cyclopentanring (3 CH_3- und 1 OH-Gruppe) nicht als vollkommen gesichert angesehen werden. Auch ENTSCHEL und KARRER zogen die Möglichkeit in Betracht, dass der Cyclopentanring in beiden Farbstoffen nicht am C_3, sondern an C_4 OH-Gruppen tragen könnte. Nunmehr gelang es uns, die Lage der OH- und CH_3-Gruppen der Cyclopentanringe eindeutig festzulegen. Capsorubinacetat



I

wurde ozonisiert, das Ozonid mit H_2O_2 behandelt und zuletzt nach Entfernung der Acetylgruppen mit Chromsäure oxydiert. Aus dem Säuregemisch wurden nach präparativer Papierchromatographie folgende Säuren kristallin dargestellt und identifiziert: 1,1-Dimethylbernsteinsäure (II), Smp. 140°C, $R_f = 0{,}37$; Trimethylbernsteinsäure (III), Smp. 149°C, $R_f = 0{,}67$; 1,1,2-Trimethylglutarsäure (IV), Smp. 110°C, $R_f = 0{,}94$; Camphoronsäure (V), Smp. 165°C, $R_f = 0{,}06$. Ausserdem wurde noch Dimethylmalonsäure (VI) nachgewiesen, jedoch reichte die Menge nicht zur Kristallisation. III, IV und V legen die Methylgruppen eindeutig fest; IV und V liefern ausserdem den Beweis, dass sich die OH-Gruppen nicht in Position 4 befinden können, und II schliesst das Vorhandensein einer OH-Gruppe an C_2 aus. Es kann so als bewiesen betrachtet werden, dass sich die OH-Gruppen in Lage C_3 und C_3' befinden.

Es erscheint wahrscheinlich, dass die OH-Gruppe des Cyclopentans auch im Capsanthin und Kryptocapsin an C_3 steht. Diesbezügliche experimentelle Befunde werden demnächst mitgeteilt.



V

[1] L. CHOLNOKY, D. SZABÓ und J. SZABOLCS, Liebigs Ann. *606*, 194 (1957).

[2] R. ENTSCHEL und P. KARRER, Helv. chim. Acta *43*, 89 (1960).

[3] M. S. BARBER, L. M. JACKMAN, C. K. WARREN und B. C. L. WEEDON, Proc. chem. Soc., Lond. *1960*, 19.

[4] L. CHOLNOKY, K. GYÖRGYFY, E. NAGY und M. PÁNCZÉL, Acta chim. Acad. Sci. hung. *6*, 143 (1955); vgl. Nature *178*, 410 (1956).

Diese Strukturbefunde fügen sich zwanglos in unsere bereits mitgeteilte Theorie, die annimmt, dass Capsorubin über Violaxanthin, Capsanthin über Antheraxanthin aus Zeaxanthin gebildet werden[4].

L. Cholnoky und J. Szabolcs

Chemisches Institut der Universität Pécs (Ungarn), 17. August 1960.

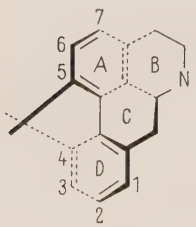
Summary

By oxidative degradation of capsorubin, it could be demonstrated that the methyl groups are in position 1, 1, 5 (1′, 1′, 5′) and the OH-group is attached to carbon atom 3 (3′). An analogous structure may be assumed for capsanthin and cryptocapsin.

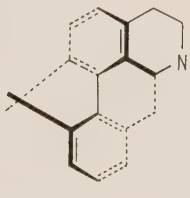
The Conformation of 4.5-Substituted Aporphine Alkaloids

In connection with some work presently in progress in the field of aporphine alkaloids[1], the question arose whether the biphenyl system involved in these bases makes a distinct contribution to the optical activity of the molecules. That this is indeed the situation, at least in some cases, is shown by the fact that in every instance in which substituents are present at both position 4 and 5, the magnitude of the optical rotation, regardless of the solvent in which it was measured, has been found to be either in the + 200° or – 200° range. On the other hand, aporphines where position 4 is unsubstituted[2] exhibited much lower rotations as indicated in Table I.

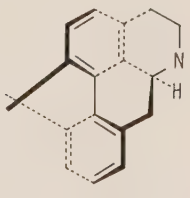
This optical phenomenon can be satisfactorily explained by assuming that in the case of the 4.5-substituted aporphines the biphenyl system, with two possible conformations such as (I) or (II), makes a substantial positive or negative contribution to the rotation of the molecules. Furthermore, a study of La Pine molecular models of 4.5-substituted aporphines indicated an intrinsic limitation in the rigid conformation of these alkaloids. Thus, if the hydrogen on the asymmetric carbon adjacent to the basic nitrogen is α (below the plane), the substituents on the A ring are β (above the plane), as indicated in (III), and *vice versa*. In other words a stereochemical situation such as existing in (IV) is forbidden for essentially the same reasons that an arrangement such as in the biphenyl (V) does not exist.



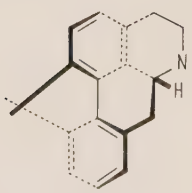
(I)



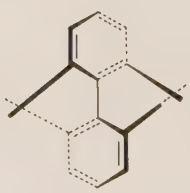
(II)



(III)



(IV)



(V)

Table I

Rotations of Some 4.5-Substituted Aporphines[3]		*Rotations of Some Aporphines Unsubstituted at Position 4*	
Corydine	+205° ($CHCl_3$)	Glaucine	+113° (EtOH)
Isocorydine	+195° ($CHCl_3$)	Boldine	+ 73° (EtOH)
Corytuberine	+282° (EtOH)	Laureline	– 99° (EtOH)
Bulbocapnine	+237° ($CHCl_3$)	Roemerine	– 80° (EtOH)
Laurepukine	–222° ($CHCl_3$)	Actinodaphnine	+ 33° (EtOH)
Isothebaine	+285° (EtOH)	Thalicmidine[4]	– 84° (EtOH)
Pukateine	–220° (EtOH)	Nuciferine[5]	–158° (EtOH)
Thalicmine[4]	+255° (EtOH)		

Table II

UV of 2.3-Substituted Aporphines	λ_{max} mμ log	λ_{max} mμ log	λ_{max} mμ log
N-Methyllaurotetanine[6]	303 (4.2)	283 (4.2)	220 (4.6)
Boldine[6]	303 (4.2)	284 (4.2)	220 (4.6)
Dicentrine[7]	310 (4.3)	282 (4.2)	—
Laurifoline Chloride[8]	307 (4.14)[a]	281 (4.04)[a]	227 (4.48)[a]
Glaucine	302 (4.17)[a]	281 (4.20)[a]	—

UV of 4-Substituted Aporphines	λ_{max} mμ log	λ_{max} mμ log	λ_{max} mμ log
Isocorydine[6]	302 (3.8)	268 (4.2)	220 (4.6)
Norisocorydine[6]	303 (3.8)	268 (4.2)	220 (4.6)
Pukateine Hydrochloride[9]	303 (4)	268 (4.1)	220 (4.4)
4,5,6-Trimethoxy-aporphine[10]	300 (3.9)	270 (4.1)	230 (4.1)
Bulbocapnine[10]	308 (3.6)	270 (3.9)	—
Menispermine Chloride[11]	303 (3.76)[a]	270 (4.13)[a]	223 (4.6)

[a] These values are exact values; all others are estimated values obtained from UV charts reproduced in articles.

[1] Supported by Grant No. B-1403 from the National Institutes of Health.

[2] All naturally occurring aporphines have positions 5 and 6 substituted.

[3] Unless otherwise indicated these values were obtained from R. H. F. Manske, The Alkaloids (Ed. by R. H. F. Manske and H. C. Holmes, Academic Press Inc., New York, N. Y. 1954), p. 119.

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[9] E. Schlittler and A. Lindenmann, Helv. chim. Acta *32*, 1880 (1949).

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[11] M. Tomita and T. Kikuchi, Pharm. Bull., Japan *3*, 100 (1955).

[12] An interesting observation made in the course of comparing ultra-violet values is that if both positions 3 and 4 are unsubstituted, such as in xylopine, stephanine, and crebanine, the peak in the 303–310 mμ region shifts to the 310–320 mμ region, and appears only as a shoulder.

[13] S. Goodwin, J. N. Shoolery, and L. F. Johnson, Proc. chem. Soc. *1958*, 306.

The above conclusions on the nature of the biphenyl system were strengthened by a correlation of the ultraviolet spectra of the aporphines (Table II). Natural aporphines substituted at position 4, or at 3 and 4, exhibited a central peak at 268–270 mμ, while 2,3-substituted aporphines exhibited a similar peak around 282 mμ. Furthermore, both of these types of aporphines showed a peak in the 303–310 mμ region, but the intensity of this peak is log 4 or less than log 4 when position 4 is substituted, and around log 4.2 when this position is free. These variations again are a reflexion of the rigid and strained biphenyl system existing in the 4-substituted aporphines[12].

Finally, Goodwin, Shoolery, and Johnson[13], on the basis of a study of the nuclear magnetic resonance spectra of the aporphines dicentrine and bulbocapnine methyl ether conjectured that the observed non-equivalence of the methylenedioxy hydrogens (quadruplet instead of singlet) could be explained by either of two views: (a) the aromatic rings are oscillating about the bond connecting them from one non-coplanar conformation to another, but the residence time in one conformation is very much greater; or (b) the relative position of the aromatic rings is fixed in some non-coplanar conformation. The above discussion makes it clear that, at least in the case of the 4.5-substituted aporphines, it is view (b) that is correct.

M. Shamma

Department of Chemistry, Pennsylvania State University, University Park (Pennsylvania), May 19, 1960.

Résumé

Par l'étude des rotations optiques, des spectres de l'ultraviolet, et des modèles moléculaires des alcaloides de l'aporphine, il a été démontré que dans les aporphines substituées en positions 4 et 5 le système biphényl est rigide et tordu.

Physikalisch-chemische Untersuchungen eines komplexen Eiweisses: das lösliche Eiweiss der Augenlinse, α-Crystallin

Die physikalisch-chemische Untersuchung des α-Crystallins gibt ein nahezu klassisches Beispiel davon, wie man durch Kombination von grundsätzlich verschiedenen Methoden Einsicht in die komplizierte Natur von Proteinen gewinnen kann, auch dann, wenn die Komplexität so gross ist, dass exakte Bestimmungen von Molekulargewicht und -form, Diffusionskonstante usw. unmöglich sind.

Man lese zum Beispiel die Verhandlungen von Lundgren und Williams[1,2], worin ausführlich über die Ergebnisse der Sedimentations- und elektrophoretischen Analyse berichtet wird.

Die löslichen Proteine der Augenlinse der Vertebraten sind nicht nur vom rein chemischen Standpunkt aus wichtig, sondern auch für immunbiologische, Induktions- und ontogenetische Differenzierungsversuche[3–6].

Die Augenlinse von höchstwahrscheinlich allen Vertebraten enthält α-Crystallin[7]. Die serologische Identität ist bei Säugetieren, Reptilien, Amphibien und Vögeln dargelegt[8,9]; beim Fisch ist dies noch einigermassen zweifelhaft. Es ist sogar möglich, dass der *Octopus vulgaris* α-Crystallin in der Augenlinse enthält[10].

Extraktion der Augenlinse von Vertebraten mit verdünnten Pufferlösungen bei einem pH über 7.0 ergibt eine trübe Lösung, die α-Crystallin, andere organspezifische Eiweisse (zum Beispiel β-Crystallin) und ein unlösliches Eiweissgemisch (Albuminoid genannt) enthält[11,12]. Die Peripherie der Linse dispergiert sehr leicht. Da der Linsenkern in der Hauptsache unlösliche Proteinaggregate enthält, wird bei den Versuchen nur die Linsenperipherie extrahiert[13,14]. Man kann α-Crystallin von den anderen löslichen Crystallinen trennen durch Fällung am isoelektrischen Punkt (pH 5.0), wobei die anderen Proteine in Lösung bleiben.

Der Niederschlag von α-Crystallin ergibt nach Auflösen in schwach alkalischem Milieu eine klare Lösung, die bei *Elektrophorese* nur *einen* Gradient bildet[15,16] (Fig. 1a). Bei *Sedimentation* dieser Lösung entstehen aber *zwei* deutliche Gradienten mit Sedimentationskonstanten von $S_{20} \sim 15$ und $S_{20} \sim 7.5$ (Fig. 1b).

Man kann also annehmen, dass α-Crystallin (mindestens) aus zwei Komponenten α_A und α_B besteht, welche bei der Elektrophorese nur *einen* Gradient bilden.

Die Aminosäurezusammensetzung von isoelektrisch präzipitiertem α-Crystallin ist von verschiedenen Forschern untersucht worden[16–21]. Diesen analytischen Angaben zufolge scheint die Zusammenstellung der α-Crystallinfraktion, unabhängig von der Darstellungsweise, konstant zu sein. Sedimentationsmessungen deuten aber an, dass das Konzentrationsverhältnis der zwei α-Komponenten bei verschiedenen Präparaten stark wechselt. Wenn also diese Komponenten verschiedene Aminosäurezusammensetzungen besässen, müssten die Zusammenstellungen verschiedener Präparate ebenfalls stark voneinander abweichen. Dieses scheint jedoch nicht der Fall zu sein. Man kann daher wohl mit grosser Wahrscheinlichkeit annehmen, dass die Aminosäurezusammensetzung der zwei α-Komponenten die gleiche ist.

Die betreffenden Untersuchungen wurden ausgeführt unter der Ägide der Niederländischen Stiftung für Chemische Forschung (S. O. N.) mit Unterstützung der Niederländischen Organisation für reinwissenschaftliche Forschung (Z. W. O.).

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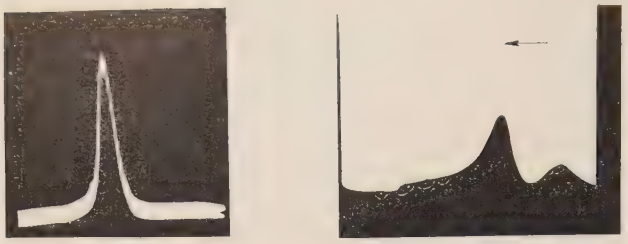
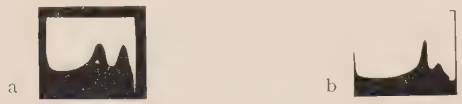


a b

Fig. 1. a) Elektrophorese-Gradient von isoelektrisch präzipitiertem α-Crystallin. 165 V, 12 mA, 8°C, 40 min, 0,1 *M* NaOAc, 0,05 *M* Glycin, pH 7,8 (NaOH). b) Sedimentations-Gradient vom gleichen Präparat. 50000 rpm, 1500 sec, 17°C, 15° ∠, 1,1% Conc.



a b

Fig. 2. a) und b) Sedimentationsgradienten von ein- und zweimal präzipitiertem α-Crystallin. 42000 rpm, 1500 sec, 45° ∠, 1,2% Conc., Temp.: a: 21,1°C; b: 20,0°C.

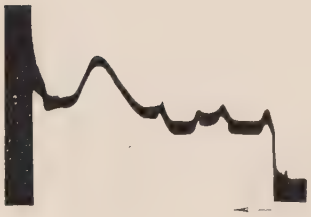


Fig. 3. Sedimentationsgradient von zweimal isoelektrisch präzipitiertem α-Crystallin, wieder aufgelöst in 0,1 *M* NaOAc, 0,05 *M* Glycin, pH 7,1; 1,4% Conc.; 47,760 rpm, 2820 sec, 12,1°C, 45° ∠.

Wenn man die Lösung von α-Crystallin bis pH 2.5 ansäuert, entsteht eine klare Lösung, die in der Ultrazentrifuge nur *einen* Gradienten bildet mit einer Sedimentationskonstante von $S \sim 1$. Dies bedeutet, dass beide Komponenten zu einer einzelnen Verbindung dissoziieren. Dieses Spaltungsprodukt besitzt noch alle typischen Eiweisseigenschaften und ist serologisch identisch mit α-Crystallin.

Wenn man das isoelektrische Präzipitat bei pH 7.8 auflöst und diese Lösung wiederholt durch Ansäuern bei pH 5.0 präzipitiert und diesen Niederschlag erneut bei pH 7.8 auflöst, so ändert sich das Sedimentationsdiagramm. Die Konzentration der schnellen Komponente nimmt ab, die der langsamen Komponente nimmt zu. (Fig. 2a und 2b.)

Nach langdauernden Arbeitsgängen sowie nach fortgesetzter Sedimentation ergibt sich öfters eine Aggregation der α_B-Komponente: Figur 3.

Der verbleibende Hauptgradient rührt von der α_A-Komponente her. Dies weist darauf hin, dass der Unterschied zwischen den zwei Komponenten in der Form der Teilchen zu suchen ist, dass also die (native) α_A-Komponente entfaltet worden ist.

Die gleichmässige Abflachung des α_A-Gradienten während der Sedimentation weist darauf hin, dass die native Komponente kugelförmige Gestalt hat. Eine exakte Messung der Diffusionskonstante ist jedoch völlig ausgeschlossen, weil wir die zwei Komponenten nicht quantitativ trennen können, so dass es unmöglich ist, eine homogene Lösung der α_A-Komponente zu erhalten.

Rein theoretisch konnten wir aber beweisen[22], dass eine Lösung, welche kugelförmige und gestreckte Teilchen von gleichem Molekulargewicht enthält, wobei das Achsenverhältnis der gestreckten Teilchen $1_1/1_0 = 20$ beträgt, zwei Gradienten in der Ultrazentrifuge bildet, deren Sedimentationskonstanten sich wie 1 zu 2 verhalten, während sich bei der Elektrophorese dieser Lösung nur *ein* Gradient bilden muss, wie dies beim α-Crystallin der Fall ist. Das es sich beim α-Crystallin um einen Entartungsvorgang handelt, worin mehr oder weniger globuläre, native Teilchen einer Streckung unterzogen werden, ehe die weitere Denaturierung fortschreiten kann, wird durch die obrigen theoretischen Überlegungen wahrscheinlich gemacht und wird unterstützt durch die Kristallisationsversuche von Wood *et al.*[23]. Diese Forscher arbeiteten mit Präparaten, welche durch ihre Bereitungsweise entartete Linseneiweisse enthielten. Die Nadelstruktur der «α»$_B$-Kristalle weist auf eine langgestreckte Form dieser entarteten Eiweissteilchen hin.

Die bisherige physikalisch-chemische Untersuchung des Linseneiweisses α-Crystallin ergibt ein Bild, das, obgleich noch hauptsächlich qualitativ, doch den Weg zeigt, nach welchem weitere quantitative Untersuchungen erfolgen müssen, um die Struktur dieses besonders interessanten Eiweisses zu klären.

W. F. Bon

Laboratorium für Biochemie und Toxikologie der Universität Amsterdam (Niederlande), 7. April 1960.

Summary

Ultracentrifugal and electrophoretic investigations of the eye lens protein α-crystallin show the probability that this protein exists in the lens and in its solutions (between pH 3.5 and 9.5) in two sub-units only differing in shape: a globular and a stretched unit.

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The Investigation of the Action of Ultrasound on some Proteid Substances. Part II.[1] The Action of Ultrasound on Diastase

Numerous publications which have appeared in recent years[2] show that in addition to engineering, medicine and biology, chemistry and particulary biochemistry represent a broad field of application for ultrasound. Its action on ferments has been established on oxidases[3], blood catalase[4], yeast ferments[5], pepsin[6], salivary diastase[7], and tripsin[8].

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Time of ultrasonic treatment	Enzyme activity in Willstätters units	pH values of solution
0 min	9.9×10^{-4}	5.50
5 min	8.5×10^{-4}	5.50
10 min	7.0×10^{-4}	5.20
15 min	5.6×10^{-4}	5.05
20 min	4.8×10^{-4}	4.90
30 min	2.7×10^{-4}	4.70

The results obtained show that the activity of 0.5% diastase solutions and the pH values have been decreasing gradually but regularly.

These investigations are of significance for application of ultrasound in practical medicine as well. The mechanism of the action of ultrasound being still insufficiently explained, it is necessary to be careful when applying it for therapeutic purposes. Just for that reason, the investigation of its action on substances contained in human body is of great importance, since it offers the possibility of avoiding mistakes and accidents when applying ultrasound for therapy. We have investigated the action of ultrasound on diastase solution (0.5% B. D. H. make) and in each experiment we have used 10 ml of it.

The ultrasound of frequency 3 Mc/s and intensity of 1.5 W/sq.cm. has been obtained from a Yugoslav make of generator. The solutions have been placed in a flask, the bottom of the flask being made of thin gum. The samples were exposed for the period of 0, 5, 10, 20, and 30 min respectively. During the exposure the solutions have been cooled in tap water in such a way that the temperature has never been allowed to rise above 30°C. The activities of the solutions were determined by Willstätter's method[9] 5 h after the treatment. The pH values were measured immediately after exposure (Table).

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Zusammenfassung

Die Ultraschallwirkung auf Diastase wurde an deren Aktivitäts- und pH-Änderung untersucht. Verschieden lang beschallte, 0.5%ige wässerige Diastaselösungen ergaben stufenweises und regelmässiges Absinken der Aktivitäten und pH-Werte.

The Inhibitory Effect of 6-Azauracil Riboside on the Multiplication of Vaccinia Virus

6-Azauracil, and particularly its riboside, are known to inhibit the growth of a number of microorganisms[1,2] and experimental tumours[3–5]. We now wish to report tests on the anti-virus activity of the two compounds.

In our screening test (diffusion of the tested substance through agar using Dulbecco's plaque method for virus giving a positive cytopathogenic effect) 6-azauracil proved inactive but its riboside exhibited notable activity on the multiplication of vaccinia virus compared with benzimidazole (Figures 1–3).

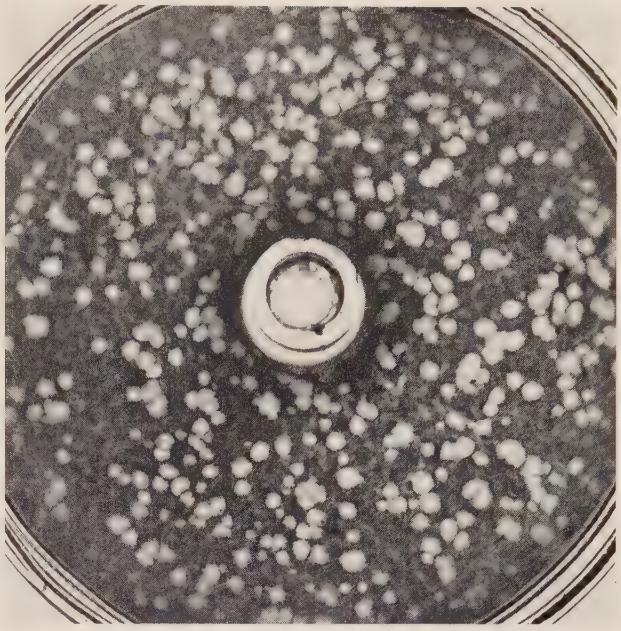

Fig. 1. 6-Azauracil (4.4×10^{-1} *M*, incompletely dissolved). No zone of inhibition or toxicity.

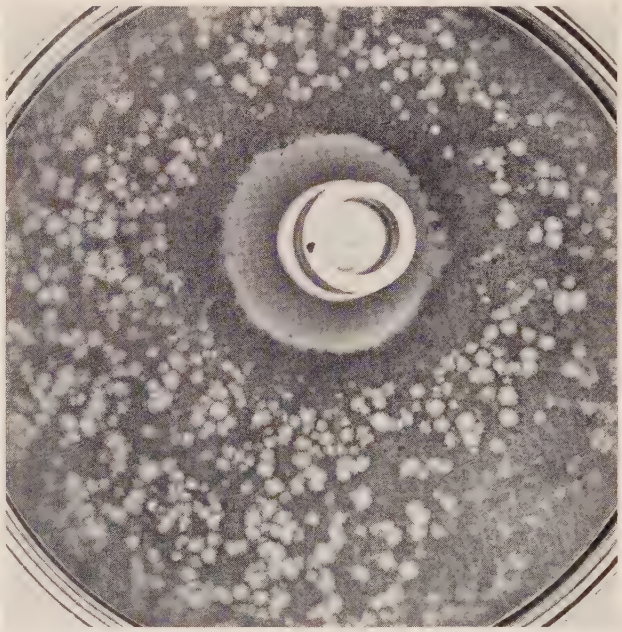

Fig. 2. Benzimidazole (8.5×10^{-5} *M*, Fluka, Switzerland). Zone of toxicity around the cylinder and further away narrow zone of inhibition.

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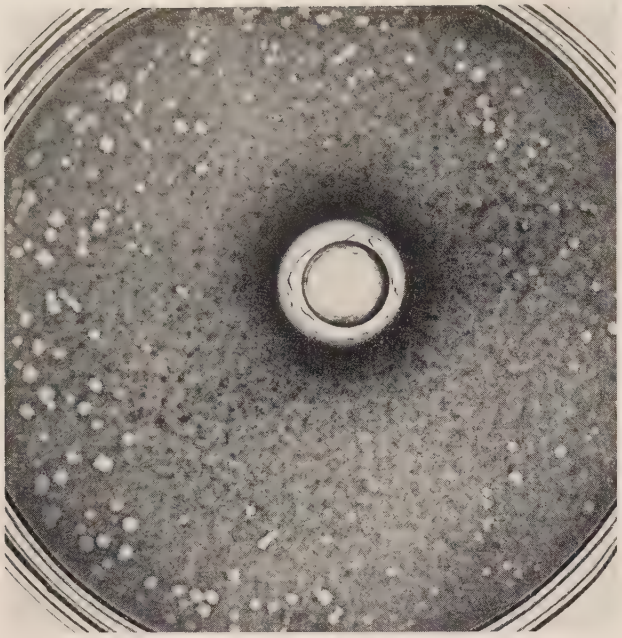

Fig. 3. 6-Azauracil riboside[6] (2.0 M). Large zone of inhibition without zone of toxicity.
The cultures were seeded with approximately 500 plaque-forming units of vaccinia virus; the cylinders were charged with 0.05 ml of solution of the substance tested; dishes of 4 cm diameter were employed.

A quantitative evaluation of the effect of 6-azauracil riboside on the multiplication of vaccinia virus was performed, using the method of Overman and Tamm[7] with the modification that the ineffectivity test was carried out by Dulbecco's plaque method on chick fibroplast cultures. In the toxicity tests, the method and criteria of Tamm[8] were adopted. 6-Azauracil riboside has very low toxicity: only concentrations as high as 1.75×10^{-1} M bring about changes in the curling up of a piece of chorioallantoic membrane characterised by Tamm as 2 +. On the other hand, a 1.2×10^{-2} M concentration of 6-azauracil riboside causes a 75% inhibition of the multiplication of vaccinia virus. Its selectivity (ratio of toxicity to virus inhibiting concentration) is thus about 14, i. e. sufficient to permit its use in amounts not toxic for the animal host.

6-Azauracil riboside has no direct inactivating effect on vaccinia virus, as shown by incubation of the virus with 4×10^{-2} M 6-azauracil riboside for 3 h at 35°C (Table).

6-Azauracil riboside was found to have no effect on influenza virus type A strain PR8, when tested by the method of Tamm *et al.*[9], and against Newcastle disease virus (NDV) and Eastern equine encephalomyelitis virus (EEE), when tested by the plaque method.

Number of Plate Forming Units (PFU)/ml in Presence of 6-Azauracil Riboside

h	Medium + 6-azauracil riboside[a]	Blank
0	2.8×10^5	2.8×10^5
3	1.5×10^5	2.4×10^5

[a] The concentration of 6-azauracil riboside was 4×10^{-2} M which corresponds to 99% inhibition in the membrane culture test.

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Zusammenfassung

Die Hemmwirkung von 6-Azauracilribosid auf die Vermehrung des Vaccina-Virus in Gewebekulturen wurde festgestellt. Die Substanz blieb gegen andere Viren (Influenza A, NDV, EEE) wirkungslos.

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A Paper Strip Electrophoretic Examination of the Action of Dextran in Colloidal Solution on Human Serum

Sulphated polysaccharides applied *in vivo*, as heparin and dextran sulphate, produce a clearing effect on human and animal lipaemic serum[1–7], and an accelerating effect on the electrophoretic mobility of the serum lipids migrating with the β-globulins and albumins[1,5,8,9].

These sulphated polyanions interact with serum *in vitro* forming an insoluble complex with the lipids migrating electrophoretically with the β-globulins[10–13]. This insoluble complex also contains proteins.

In the present paper, a preliminary investigation of a possible effect of dextran in colloidal solution on turbidity of normal and lipaemic human sera is tried. A possible action of dextran in colloidal solution on electrophoretic mobilities of serum lipids was also examined. The experiments were performed with human sera by applying dextran *in vivo* and *in vitro*.

Materials and Methods. The dextran used in infusion *in vivo* and experiments *in vitro* was 'Macrodex' from Pharmacia Uppsala Sweden in a 6% colloidal solution. The infusion was given to healthy males and females during 2 h at a rate of 210 mg/kg/h. In the experiments *in vitro* 0.30

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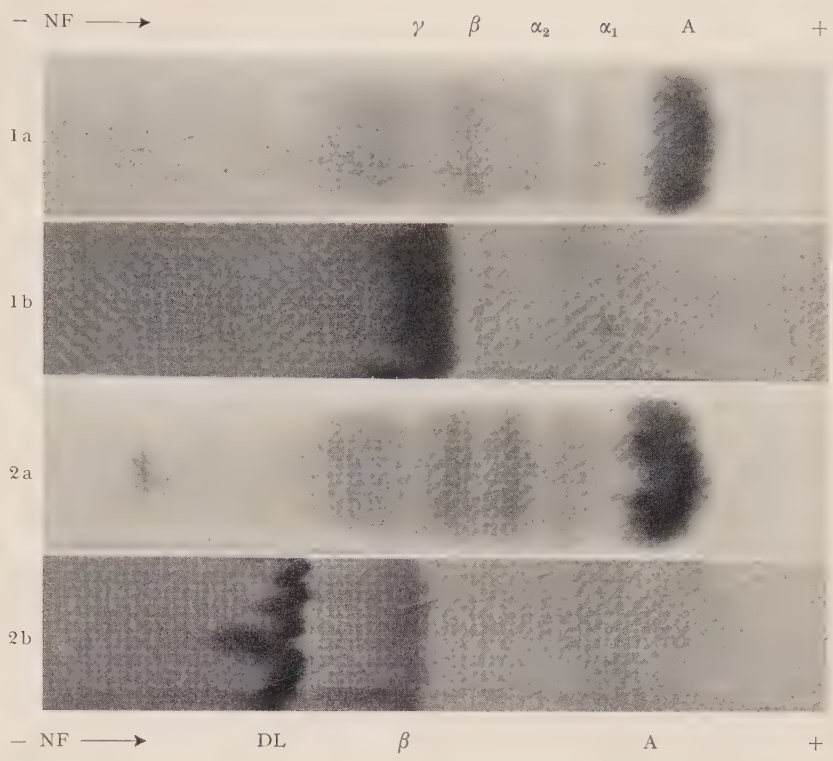
volumes of the colloidal solution of dextran was added to one volume of serum. The turbidity of serum was estimated at 590 mμ on the Zeiss Elko II photometer. The filter paper strip electrophoresis was performed in veronal Na-acetate and in phosphate buffer at pH 8.6 in a potential gradient of 3–4 V/cm for 18 h. The filter paper used was Whatman No. 1. The lipid patterns were stained with Sudanblack B and Oil Red 0, the protein patterns with Amidoblack 10B and Bromphenolblue.



The effect of dextran in colloidal solution on lipids and proteins as recorded by paper strip electrophoresis.

1. Normal serum. 2. The action of dextran on the same serum. NF = neutral fats; DL = the dextran-lipid fraction. a = protein staining. b = lipid staining.

Results. In all experiments performed *in vivo* and *in vitro* (10 cases), dextran in colloidal solution exerted no change in turbidity of fasting and lipaemic human sera. On the contrary, in all the experiments performed, *in vivo* and *in vitro* with lipaemic or fasting sera, dextran in colloidal solution changes the lipid patterns in paper strip electrophoresis. A new lipid fraction appears with a slower electrophoretic mobility than any known protein or lipid fraction. It appears between the starting point and the γ-globulins. We suggest calling this new fraction the dextran-lipid fraction (DL-fraction). The Figure represents a typical result of the experiments. The appearance of the DL-fraction obviously diminishes the intensity of the lipid pattern migrating with the mobility of the β-globulins. The protein patterns seem not to be altered by the action of dextran. It seems to us that the DL-fraction contains no proteins but an appreciable amount of dextran. Thus we assume that this fraction represents a dextran-lipid complex. Further work on the subject is in progress and will soon be published in detail elsewhere.

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Zusammenfassung

Eine 6%ige kolloidale Dextranlösung, *in vivo* infundiert oder dem Serum *in vitro* zugesetzt ändert dessen Lipidogramm. Zwischen Startpunkt und γ-Globulinen entsteht eine neue mit DL bezeichnete Fettfraktion. Im Verlauf der Experimente unterblieb eine Trübung des Serums und Änderung des Elektrophorese-Proteinogramms.

Serotonin Inhibition of Liver Mitochondria Swelling *in vitro*

In recent researches on mitochondrial morphology, it has been found that many substances possess the ability of inhibiting the swelling which occurs spontaneously when the particles are suspended in isotonic sucrose: such substances are for example ATP (adenosine triphosphate), Mg^{++}, versene, and K^{+}[1–3]. Other substances, such as phosphates or succinate, increase or accelerate this process of water exchange between the medium and mitochondria in relation to their metabolic activity[3–5]. Recently some neurohumoral amines have been considered for their action on mitochondrial morphology: histamine, adrenaline, and acetyl choline were found to be completely inactive *in vitro*[6]. In this note the effect of another neurohumoral amine, 5-hydroxytryptamine (HT), on spontaneous swelling of rat liver mitochondria *in vitro* has been studied. This neurohumoral amine is contained in appreciable amounts in blood platelets; and since these do not contain enzymes capable of metabolising it, and since substances have been found which influence the binding or release of HT from blood platelets, such as reserpine[7], it would seem that they merely have a function of transport with respect to HT. Walaszek and Abood, studying brain mitochondria, found that they contain a considerable

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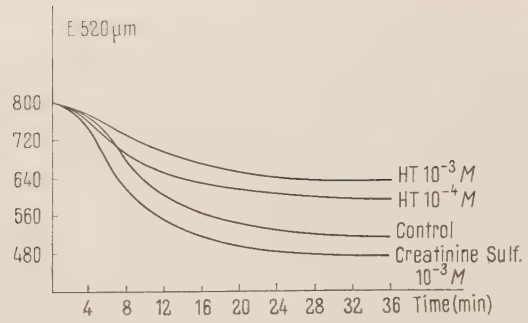

Inhibition by Serotonin on Mitochondrial Spontaneous Swelling as Estimated by the Optical Method, and Swelling Effect of Creatinine-Sulfate.

Percentage Decrease (D%) of Optical Density on Spontaneous Swelling of Rat Liver Mitochondria in 35 min

	No. of experiments	D%
Control	15	29,4 ± 6,4
Mitochondria with serotonin 10^{-3} *M*	10	20,8 ± 4,5
Mitochondria with serotonin 10^{-4} *M*	7	22,3 ± 5,1
Mitochondria with creatinine-sulfate 10^{-3} *M*	3	32,7 ± 5

amount of HT. In addition they observed that reserpine has an effect on the serotonin of these mitochondria which is parallel to that demonstrated on blood platelets[8]: it therefore seemed interesting to see whether this neurohumoral amine influenced mitochondria morphology.

Mitochondria were prepared from liver of albino rat, Wistar strain (150–200 g) body weight) in 0.25 *M* sucrose, containing 0.02 *M* Tris-chloride, pH 7.4 at 4°C, by the method of Schneider[9]. 1 ml of the final concentrated suspension of mitochondria was equivalent to 0.25 g of fresh liver. Changes in extinction at 520 mµ read in a Beckmann model DU spectrophotometer, were taken as measure of swelling as described by Cleland[1]. Each test tube contained 3 ml of 0.25 *M* sucrose, buffered with 0.02 *M* Tris-chloride, pH 7.4: HT was added to the sample examined. Then 0.3 ml of the mitochondria suspension were quickly added, the tube was shaken to mix its contents and the optical density was determined. Usually the first reading was taken within 20–30 sec after mixing and then at given times up to 35 min. The zero time or initial optical density was obtained by extrapolation. The whole experiment was made within 75 min from the killing of the animal.

The 5-hydroxytryptamine-creatinine sulfate (it is the only salt available) used was supplied by Sigma and Roche Laboratories. The results are given in the Table: a significant difference was observed in the % decrease of optical density in samples containing HT; the Figure gives the effect of serotonin on spontaneous mitochondrial swelling at two different concentrations. We also verified the effect of creatinine-sulfate on mitochondria: it was found that it has a swelling effect and is therefore an antagonist to HT.

Consequently it appears that serotonin has a protective action on mitochondria swelling *in vitro*. Further studies are being carried out in order to see if there is a correlation between ATPase activity and protective action of serotonin. This is supported by the fact that the concentration of HT in blood platelets depends on the ATP present[10] (according to Born[11], serotonin would actually be bound to ATP as a complex) and that inhibitors of HT binding by mitochondria such as reserpine, dibenamine, and phenylether, inhibit oxidative phosphorylation[9].

Anna Casu

Istituto di Patologia Generale dell'Università di Genova (Italy), June 1, 1960.

Riassunto

È stato studiato l'effetto della serotonina sul rigonfiamento dei mitocondri *in vitro:* si è trovato che la serotonina protegge i mitocondri dal rigonfiamento spontaneo che si ha quando vengono sospesi in soluzione isotonica di saccarosio.

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The Kinetic of Induction of Plaque Formation in Cell Monolayers by Ribonucleic Acid from Poliovirus

We have published previously[1] a test system for quantitative studies on the infectivity of isolated ribonucleic acid (RNA) from poliovirus. The method was developed to provide a tool for physico-chemical analysis of biological active RNA, in a manner comparable to the analysis of desoxyribonucleic acid (DNA) carried out with the aid of studies on transforming DNA. Other investigators have already applied this method for further characterization of infectious RNA[2,3]. It was of interest to learn more about the basic mechanism of the RNA test system. We present here results on the kinetic of plaque induction in cell monolayers by RNA.

The methods used for preparation and assay of RNA are essentially the same as those described previously[1]. Poliovirus type I strain Mahoney is used for isolation of RNA. The virus suspensions are obtained partially purified from the Cutter Laboratories. Highly purified poliovirus suspensions are prepared in our own laboratory according to the method of Levintow and Darnell[4]. The experimental results obtained are independent of the virus source.

The solutions for washing the cell monolayers and the RNA solution are incubated in a water bath at the stated temperatures. The cell monolayers are kept at these temperatures for 20 to 30 min before seeding with the RNA.

I acknowledge with thanks the capable technical assistance of Mrs. O. Van Damme, Mrs. S. Koenig and Mrs. G. Lange. I am indebted to Dr. K. Sprunt for communication of results and helpful discussions.

This work has been supported by grants from the National Foundation and the Deutsche Forschungsgemeinschaft.

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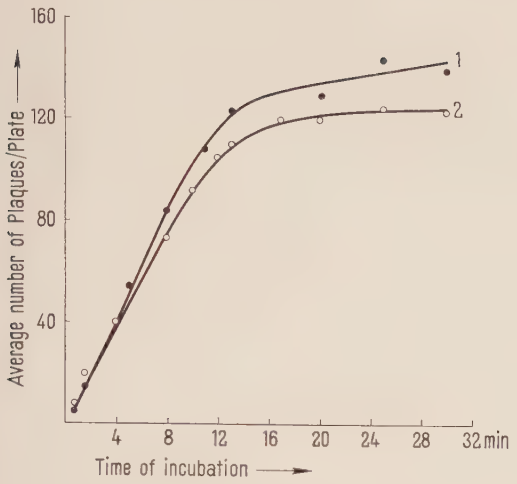

Kinetic of induction of plaque formation in cell monolayers by isolated RNA from poliovirus (average of three experiments), Curve 1 at 36°C, Curve 2 at 20°C.

The interaction of the RNA with the cells is stopped by applying the agar overlay or by washing with physiological saline (Hanks solution). Identical results are obtained with both procedures.

The results of our experiments are shown in the Figure. The interaction of viral RNA with the host cell leads to a rapid initial increase of infected plaque forming units during the first 8 to 10 min after seeding and thereafter to an ever-decreasing rate of further induction of cells to plaque forming units. The rate of plaque induction by RNA does not differ significantly when cell monolayers are incubated at 20° or 36°C after seeding, and an almost identical number of plaques/plate is obtained by a given sample of RNA at these temperatures.

We have also followed the induction of plaque formation by RNA at 0° to 4°C. The results have been erratic from one experiment to another. In all experiments the increase in the number of plaques between 0 and 20 min after seeding was somewhat lower than at 20°C, but otherwise identical with respect to the rate of uptake. The RNA infectivity at 4°C was variable over a wide range from one experiment to another and showed an infectivity of the order of 1 to 70% of its infectivity in parallel experiments at 20°C. No explanation can be offered so far for the erratic results obtained at 4°C.

The time-dependent differences in the rate of induction of plaques by RNA in cell monolayers are understandable by either one of the following assumptions: (1) The cells are sensitive to RNA only for a short interval after exposure to hypertonic saline and become less and less sensitive with time after seeding. Not all potentially infective RNA is taken up by the cells according to this hypothesis. (2) The cell monolayers are not free from RNAase or RNA inhibitors. (3) The isolated RNA from poliovirus is heterogeneous with regard to the ability to interact with cells.

Experiments are being undertaken in our laboratory to prove or disprove these assumptions. Results obtained so far strongly favour hypothesis 1.

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Zusammenfassung

Die Kinetik der Induktion von Plaques in Einschichtenzellkulturen wurde bei 20° und 36°C bestimmt. Es nimmt die Zahl der durch RNS induzierten Plaques in den ersten 8 bis 10 min nach Kulturbeimpfung schnell und dann allmählich langsamer zu. Die möglichen Ursachen dieses Verhaltens werden diskutiert.

Thermische Denaturierung von Kaltblüter-Enzymproteinen

Die Adenosintriphosphatase aus Karpfenmuskulatur zeigt eine bemerkenswerte hohe Hitzelabilität (Partmann[1]). Versuche, eine Hitzedenaturierung bei der Reinigung von Kathepsin aus Dorschmuskulatur anzuwenden, waren ohne Erfolg (Siebert und v. Malortie[2]). Der Dorsch, *Gadus callarias* (= *morrhua*) L., lebt üblicherweise bei einer Wassertemperatur von + 5 bis + 10°C. Es erhebt sich daher die Frage, ob es eine generelle Eigenschaft von Kaltblüter-Enzymproteinen ist, dass sie bereits bei wesentlich niedrigeren Temperaturen denaturiert werden als es der allgemeinen Erfahrung mit Warmblüterenzymen entspricht. Die nachstehend beschriebenen Versuche wurden zur Klärung dieser Frage unternommen.

Methodik. Im allgemeinen werden thermische Denaturierungen von gelösten Proteinen so vorgenommen, dass für den Effekt sowohl die Zeitdauer der Erhitzung als auch die erreichte Temperatur wichtig sind. In den hier zu beschreibenden Versuchen hat es sich bewährt, den Zeitfaktor dadurch zu eliminieren, dass grundsätzlich die gewünschte Temperatur in einer wassergefüllten Durchströmungsapparatur während voller 60 min zur Einwirkung kam. Kontrollversuche haben gezeigt, dass im allgemeinen 10–15 min, höchstens jedoch 30 min ausreichen, um ein bestimmtes Denaturierungsausmass herzustellen, das sich bei Ausdehnung der Erhitzungszeit auf 60 min nicht mehr wesentlich ändert. Die nach so definierter Hitzeeinwirkung messbare Enzymaktivität wurde in % des Ausgangswertes ausgedrückt und gegen die angewandte Temperatur graphisch aufgetragen. Als Kriterium der thermischen Resistenz diente die durch Extrapolation von mindestens 4 – auf einer Geraden liegenden – Messpunkten erhaltene Temperatur, bei der 50% der Anfangsaktivität des betreffenden Enzyms zerstört sind. Als Untersuchungsmaterial wurde in fangfrischem Zustand (auf See) tiefgefrorenes Dorschfilet benutzt, das zur Extraktion der Enzymproteine der Glykolyse mit 1%iger KCl-Lösung, zur Extraktion proteolytischer Enzyme mit 1%iger LiBr-Lösung homogenisiert wurde. Aktivitätsbestimmung der Enzyme: Kathepsin nach Laskowski[3], Glycylglycindipeptidase nach Smith[4], Ninhydrinreaktion nach Moore und Stein[5], Isocitricodehydrogenase nach Siebert *et al.*[6], Milchsäuredehydrogenase nach Bücher

Die Untersuchungen wurden dankenswerterweise durch eine Beihilfe des Bundeswirtschaftsministeriums Bonn unterstützt. Herrn Degener, i. Fa. Hanseatische Hochseefischerei A.G., Bremerhaven-F., verdanken wir das Untersuchungsmaterial.

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Tab. I. Aktivität und Hitzedenaturierung verschiedener Enzyme in der Dorschmuskulatur

Enzym	Enzymquelle	Messtemperatur °C	Aktivität gesamt µMol/h/g Frischgewicht	Aktivität spezifisch µMol/h/mg Protein	Temperatur, bei der nach 1stündiger Einwirkung 50%ige Denaturierung erfolgt
Kathepsin, Rohextrakt	Dorschmuskel	37	2.9(Tyrosin)	0,17	44
Kathepsin, teilgereinigt	Dorschmuskel	37	—	5,3	52
Glycylglycindipeptidase	Dorschmuskel	40	420	158	42
Glycylglycindipeptidase	Rindermuskel	40	185	35	46
Milchsäuredehydrogenase	Dorschmuskel	25	20 100	750	52
Aldolase	Dorschmuskel	25	1 250	43	41
Triosephosphatdehydrogenase	Dorschmuskel	25	4 600	180	51
Enolase	Dorschmuskel	25	3 080	128	45
Pyruvatkinase	Dorschmuskel	25	3 410	141	51
Isocitricodehydrogenase	Dorschmuskel	25	202	8,4	30

Tab. II. Q_{10}-Werte von zwei Enzymen aus Dorschmuskulatur

Messbereich in °C, der zur Berechnung benutzt wurde	Glycylglycin-dipeptidase	Kathepsin, teilgereinigt
von 37 bis 27	1,66	2,03
32 22	1,84	
27 17	1,97	1,86
22 12	2,13	
17 7	2,17	2,13
12 2	2,67	
7 – 3		2,41
–3 –13		2,45

et al.[7], Aldolase, Triosephosphatdehydrogenase und Pyruvatkinase nach Bücher *et al.*[8], Enolase nach Bücher[9], Proteinbestimmung nach Lowry *et al.*[10].

Ergebnisse. Die in Tabelle I enthaltenen Versuche zeigen, dass der Bereich 50%iger Denaturierung in sehr weiten Grenzen, zwischen 30 und 52°C, schwankt. Die von Partmann untersuchte Adenosintriphosphatase fällt mit etwa 38°C in den unteren Bereich. Gesetzmässigkeiten bezüglich der Lebensweise als Kaltblüter lassen sich aus diesen Daten daher nicht ableiten. Proteinchemische Konsequenzen hinsichtlich besonders geringer thermischer Stabilität (etwa im Gegensatz zu den hochgradig hitzeresistenten Enzymproteinen thermophiler Bakterien) können erst erörtert werden, wenn die betreffenden Enzyme in gereinigter Form vorliegen. Reinigungseffekte durch Erhitzung haben wir beobachtet bei Milchsäuredehydrogenase (2fach), Enolase (2,2fach), Pyruvatkinase (2,8fach) und Triosephosphatdehydrogenase (3,5fach), jedoch nicht weiter verfolgt. Von den verschiedenen Erklärungsmöglichkeiten, warum gereinigtes Kathepsin (Siebert und v. Malortie[2]) eine grössere Hitzeresistenz als das im Rohextrakt vorliegende Enzym aufweist, wird keine durch die bisher vorliegenden experimentellen Erfahrungen gestützt.

Wie zu erwarten, sind die thermodynamischen Qualitäten der Kaltblüterenzyme gleich denen der Warmblüterenzyme. Dies geht aus Tabelle II hervor, in der für Glycylglycin-dipeptidase und für Kathepsin aus Dorschmuskulatur die Temperaturkoeffizienten Q_{10} zusammengestellt sind. Der im Messbereich 37–27°C aus der Reihe fallende Wert für Kathepsin, der einen im Verhältnis zu geringen Umsatz bei 37°C anzeigt, dürfte auf bereits beginnende thermische Denaturierung zurückzuführen sein, obwohl erfahrungsgemäss bei den hier untersuchten Enzymen Substratgegenwart vor der Hitzedenaturierung in gewissem Umfang zu schützen vermag.

G. Siebert, A. Schmitt,
R. v. Malortie und E. Adloff

Physiologisch-chemisches Institut der Universität Mainz (Deutschland), 14. Juli 1960.

Summary

The temperature, which leads to 50% reduction of catalytic activity by heat denaturation, has been determined for 8 different enzymes from cod muscle, as being in the range between 30 and 52°C. Therefore, there are no indications of a generally different heat resistance of enzymes from cold-blooded animals as compared with those from warm-blooded animals. The same conclusion is derived from calculations of Q_{10}-values, measured between + 37 and – 13°C for cathepsin and glycylglycine dipeptidase.

[7] A. Delbrück, E. Zebe und T. Bücher, Biochem. Z. *331*, 27 (1959).

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Release of 5-Hydroxytryptamine and Histamine from Neoplastic Mast Cells by Alkaline Tissue Extracts[1]

The pharmacological release of biogenic amines from a number of tissues by reserpine and related compounds is now a well-documented phenomenon[2,3]. In the intact anesthetized dog, without pharmacological intervention, 5-hydroxytryptamine (5-HT, serotonin) has been shown to be released from the gastro-intestinal tract, but the natural stimulus responsible for this release is not known[4].

[1] Aided in part by grant B-940 from National Institute of Neurological Diseases and Blindness, and in part by the James Hudson Brown Memorial Fund of Yale Medical School.

[2] B. B. Brodie, in *5-Hydroxytryptamine* (Ed. by G. P. Lewis, Pergamon Press, New York and London 1958, p. 64.)

[3] M. K. Paasonen and M. Vogt, J. Physiol. *131*, 617 (1956)

[4] C. C. Toh, J. Physiol. *126*, 248 (1954).

In this connection, the observations of Toh[5,6] that alkaline extracts of certain mammalian tissues, under *in vitro* conditions, will liberate both 5-HT and histamine from platelets (dog), are of some interest.

In using suspensions of neoplastic mast cells (mouse) instead of platelets as a source of 5-HT and histamine we have confirmed and extended the observations of Toh, in a preliminary attempt at characterizing the active principle(s).

Extracts of various tissues of the rat were prepared by a slight modification of the method of Toh[5] so that approximately 0·2 ml of extract was derived from each 50 mg of tissue. The neoplastic mast cells, harvested from the ascitic fluid of mice inoculated 7 to 10 days earlier, were centrifuged, washed and suspended in either Hank's solution[7] or a calcium-free fluid originally described by Tullis[8] and modified by Toh[5]. The cold suspension of cells (usually 1 ml containing 10×10^6 cells) and a small volume of extract (usually 0·1 ml) were mixed and incubated at 37°C for 40 min. At the end of that time the cells were separated by centrifugation, and both cells and supernatant medium were analyzed separately for their content of 5-HT (and, occasionally, of histamine). The 5-HT was extracted by means of 95% acetone as described by Amin, Crawford, and Gaddum[9] and assayed on the heart of *Mercenaria* (*Venus*) *mercenaria*. Histamine was extracted with 1 N HCl; and the centrifuged, neutralized extract was assayed on the isolated ileum of the guinea pig.

Untreated suspensions of cells, with pH adjusted appropriately, served as controls in each experiment. These always showed a small release of 5-HT (10%) at the end of the incubation procedure; and, therefore, all results are expressed as % total release minus % release found in control tubes.

A number of tissues of the rat were extracted and the extracts tested for their 5-HT releasing activity. Composite results of 5 experiments are shown in Table I.

It is clear that extracts of brain had the greatest activity. Extracts of lung, kidney, duodenum and spleen were also active; those from heart were barely active; and extracts of blood and skin were inactive. These findings agree generally with those of Toh[5,6] and of Archer[10]. Toh, however, laid special emphasis upon activity of kidney extracts, without having observed the equal or greater activity of extracts of brain.

Varying the number of neoplastic mast cells in the test suspensions from 8 to 23×10^6 cells per ml of suspending medium had no consistent effect on the % of 5-HT released by a given concentration of brain extract; however, the amount of 5-HT released varied with the amount of extract (over the range: 5 to 56 mg, i. e., the tissue equivalent, diluted to 0·2 ml with suspending medium). Results of two representative experiments are summarized in Table II.

In experiment 2, the brain extract used was one found to have relatively low activity (maximum release; 52%); however, it is evident that the extract from as little as 5 mg of brain can cause a substantial release of 5-HT. These data would indicate that an excess of extract was used in all other experiments.

The extent of release of histamine studied under identical conditions was similar to that of 5-HT. For instance, a potent brain extract which caused the release of 86% 5-HT liberated 95% of the histamine in the cells.

The release of 5-HT by extracts of brain was dependent upon temperature, but not upon pH (in the range, pH 5·0 to pH 8·0), and it increased directly with the duration of incubation. Furthermore, the active principles in the

Tab. I. 5-HT Releasing Activity of Alkaline Extracts of Various Tissues of the Rat

Tissue	Equivalent Weight Tested (mg)	5-HT Content (γ)			5-HT Release (%)
		Supernatant	Cells	Total	
Control	—	0.1	2.4	2.5	4
Brain	68	4.8	0.4	5.2	92
Lung	74	4.4	1.0	5.4	81
Kidney	59	1.0	0.3	1.3	76
Duodenum	64	0.8	0.3	1.1	73
Spleen	60	2.6	1.3	3.9	67
Heart	50	0.8	1.6	2.4	33
Blood	50	0.3	2.0	2.3	13
Skin	50	0.2	4.8	5.0	3

Tab. II. Relation of Concentration of Alkaline Extracts of Rat's Brain to Release of 5-HT from Mast Cells

Experiment	Tissue Equivalent of Extract (mg)	5-HT Content (γ)			5-HT Release (%)
		Supernatant	Cells	Total	
1	14	2.8	0.6	3.4	84
	28	2.8	0.3	3.1	92
	56	3.2	0.2	3.4	94
2	5	0.7	1.6	2.3	29
	10	0.8	1.5	2.3	33
	15	0.5	1.4	1.9	38
	20	1.1	1.2	2.3	48
	25	1.0	1.3	2.3	42
	50	1.2	1.1	2.3	52

Tab. III. Influence of Suspending Medium on Amine-Releasing Potency of Alkaline Extracts of Rat's Brain

Suspending Medium	% Release of 5-HT	% Rel. of Histamine	% Cells «Non-Viable»[a]	
			Treated	Control
Isotonic Salt	20	—	20	18
Tullis-Toh's	86	95	18	5
Ringer-Locke's	50	—	22	7
Locke-Lewis'	2	—	4	4
Hanks'	10	12	2	4

[a] As indicated by microscopic examination.

[5] C. C. Toh, J. Physiol. *133*, 402 (1956).
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[10] G. T. Archer, Nature *182*, 726 (1958).
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[14] This work was done in partial fulfillment of the requirements for the degree of Doctor of Medicine by L. T. P.
[15] Squibb post-doctoral fellow in Pharmacology; present address, Department of Pharmacology, Royal College of Surgeons, London.

extracts were stable to heat, acid and alkali, and could not be separated completely from protein by these and other precipitants, or by dialysis.

To test the hypothesis that the amine-releasing activity of the extracts might be mediated by damage to the membrane of the neoplastic mast cell, experiments were carried out in a variety of media ranging from simple isotonic salt solution to Hanks' solution (a complex salt solution containing glucose, used as the basal salt medium in the culture of neoplastic mast cells *in vitro*[11]. Table III summarizes the results of these experiments.

In those solutions containing glucose, Hanks' and Locke-Lewis', the potency of the brain extract was markedly inhibited, as was destruction of the cells. In fact, the degree of amine-release which occurred in Tullis-Toh's or Ringer-Locke's solutions could be accounted for in part by destruction of the cells. By contrast, the entire release of 5-HT produced in isotonic salt solution was directly related to cell destruction. Further experiments have served to show that the most of the antagonism to amine-release seen in Hanks' solution is attributable to the presence of glucose in this medium. Such an effect of glucose might be expected if the extracts had a non-specific damaging action on the cell membrane which could be opposed by increased metabolic activity.

The activity of these tissue extracts may be compared most closely to the action of certain monoamines and surface-active agents (e. g., saponin)[12,13]. The extracts caused some apparent damage to the cells, but lysis of the cells was not sufficient to explain the activity observed. These extracts probably did exert a surface action, but it is unlikely that purely toxic, surface-active principles would have had all the characteristics of temperature dependence, variation in tissue source, relatively low lytic activity and «dosage»-effect relationships which were observed. It is generally concluded that degradation products (probably polypeptides or simpler amines) derived from injured tissues can cause the release of 5-HT and histamine from certain storage sites.

N. J. Giarman, L. T. Potter[14], and Margaret Day[15]

Department of Pharmacology, Yale University, School of Medicine, New Haven (Connecticut), July 21, 1960.

Zusammenfassung

Alkalische Extrakte diverser Rattengewebe setzen 5-Hydroxytryptamin (Serotonin) und Histamin aus Suspensionen neoplastischer Mastzellen der Maus frei. Gehirn- und Nierenextrakte zeigen die grösste Aktivität. Zugabe von Glukose in die Nährlösung verhindert das Freiwerden der Amine. Eine vorläufige Charakterisierung des aktiven Materials ist unternommen worden.

Histamine et métaux cancérigènes

Un travail précédent a établi la fixation de l'histamine par les substances cancérigènes organiques de structure différente[1,2]. Il a paru intéressant d'élargir le champ et d'observer le comportement de l'amine avec les métaux actifs (argent, nickel, cobalt, chrome, glucinium).

En effet, lorsque histamine est au contact de divers sels de ces métaux, une base complexe prend naissance en phase aqueuse. Dans certains cas le complexe apparaît sous une forme cristallisée qui entraîne une étude minéralogique, dans d'autres cas le complexe est hydrosoluble et la méthode des variations continues de Job s'applique à l'absorption lumineuse dans l'ultra-violet[3].

L'argent s'est révélé cancérigène à divers auteurs qui implantèrent des fragments métalliques dans la paroi abdominale de rats. Les tumeurs se sont développées *in situ*[4]. L'étude physico-chimique comporte le fluorure, le nitrate, le sulfate, l'acétate et le butyrate d'Argent. Le complexe $[Ag\ Hi_2]^+$ est caractérisé quelle que soit la nature de l'anion. La constante de stabilité $K = 3 \cdot 10^{-10}$ à 20°C (Fig. 1).

Le nickel a fait l'objet d'études biologiques très poussées. Les applications de métal divisé produisent le cancer au lieu même du dépôt chez les rats et les lapins avec un certain caractère de spécificité quand le véhicule utilisé pour la poudre métallique s'est révélé strictement inactif. De plus, les cancers des voies respiratoires se sont développés nombreux, provoqués par la poussière de nickel dans l'industrie du métal[5,6]. La complexion du nickel par l'histamine a déjà été entrevue dans une étude électrochimique des solutions diluées par divers auteurs (voir réf. [7]). L'étude présente permet de caractériser les complexes $[Ni\ Hi_2]^{++}$ et $[Ni\ Hi_3]^{++}$ en solution et à l'état cristallisé[7].

Le cobalt métallique divisé, administré par injections intramusculaires dans la cuisse de rats, provoque l'apparition de tumeurs *in situ*. Les injections intra-fémorales pratiquées chez le lapin produisent des tumeurs *in situ* et au loin[8]. Les sels cobalteux forment avec histamine le complexe soluble $[Co\ Hi_2]^{++}$ déjà mis en évidence par un important travail électrochimique (voir réf. [7]). La méthode de Job confirme et complète cette étude. L'examen spectral s'étend au nitrate, au chlorure et au sulfate cobalteux.

Les particules de chrome implantés dans le fémur ou introduites dans le poumon de divers animaux (lapins, chiens) font naître des tumeurs au lieu du dépôt et à distance. La fréquence des cancers observés chez les ouvriers de l'industrie vient à l'appui de l'expérimentation[9,10]. L'étude physico-chimique donne les résultats suivant: 1° Si l'on met au contact de solutions d'histamine ($M/100$) des solutions de Cl_3Cr de même titre, on observe dans les mélanges de Job une précipitation lorsque le milieu devient alcalin. L'étude spectrale des mélanges restés clairs, donne après 10 jours de contact, une courbe où la complexion de Cr par histamine apparaît déjà (Fig. 2). 2° Dans les mélanges faits à force ionique constante $\mu = 1$, le pH reste compris entre 2,75 et 4,60 zône ou Cr^{+++} existe soluble. On caractérise le complexe $[Cr\ Hi_2]^{+++}$ la constante de stabilité $k = 5 \cdot 10^{-6}$ (Fig. 3).

Le glucinium est hautement cancérigène[11]). De nombreux cas de cancérisation chez les ouvriers en contact avec les poussières ou les vapeurs de métal ont été relevés[12–14]. Le glucinium introduit dans la peau de jeunes verrats, administré par voie respiratoire, injecté par voie sous-cutanée ou intraveineuse à des souris, à des rats et à des lapins, est retrouvé *in situ* et même trois mois après

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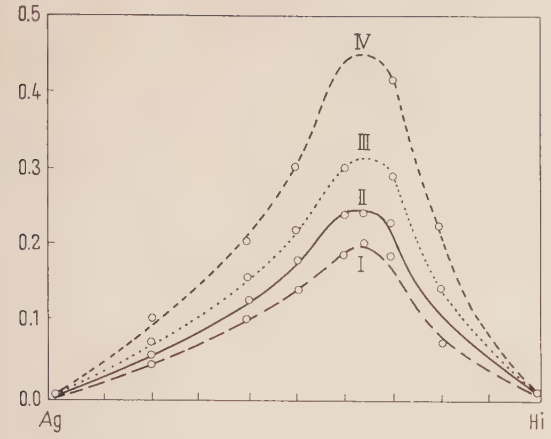

Fig. 1. Densité optique observée sous 1 cm d'épaisseur pour les mélanges de solutions équimoléculaires $M/500$ de nitrate d'argent et d'histamine.
Courbes I: $\lambda = 2650$ Å, II: $\lambda = 2600$ Å, III: $\lambda = 2550$ Å, IV: $\lambda = 2500$ Å.

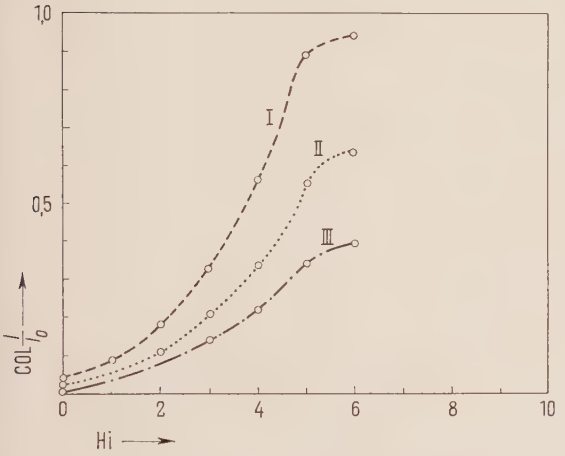

Fig. 2. Densité optique observée sous 1 cm d'épaisseur pour les mélanges des solutions équimoléculaires $M/100$ de Cl_3Cr et de histamine.
Courbes 1: $\lambda = 2700$ Å, II: $\lambda = 2800$ Å, III: $\lambda = 2900$ Å.

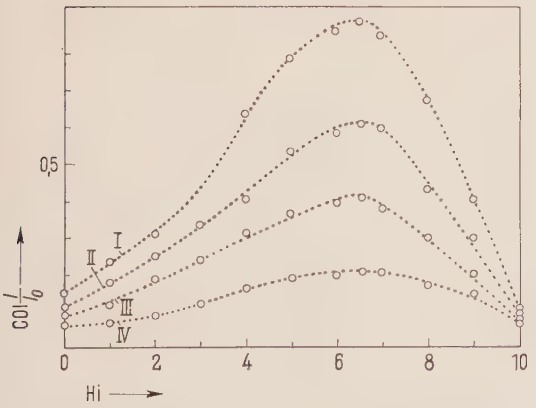

Fig. 3. Densité optique observée sous 0,5 cm d'épaisseur pour les mélanges de solutions équimoléculaires $M/100$ de Cl_3Cr et de Histamine à force ionique constante $\mu = 1$.
Courbes I: $\lambda = 2500$ Å, II: $\lambda = 2600$ Å, III: $\lambda = 2900$ Å.

une seule injection. A la suite d'un contact prolongé et sous l'action de fortes doses il apparaît dans différents organes et tout particulièrement dans les os. L'étude physico-chimique du glucinium (Be) s'apparente à celle du chrome. Si l'on fait des mélanges de solutions équimoléculaires ($M/100$) de Cl_2Be et de histamine un précipité gélatineux de $Be(OH)_2\ n\ H_2O$ se forme dans les milieux alcalins. Après 24 h de contact, les mélanges hétérogènes une fois dévantés, l'étude spectrale accuse une particularité. A force ionique constante, $\mu = 0,4$ et dans la zone des pH inférieurs à 5 zone ou Be^{++} existe soluble, aucun précipité n'apparaît et les écarts à la loi d'additivité atteignent un maximum net correspondant à l'ion complexe $[BeHi_2]^{++}$.

Il est vraisemblable qu'en solution, un chélate prend naissance avec certains métaux, engageant à la fois la chaîne et le noyau imidazole de l'amine (Fig. 4).

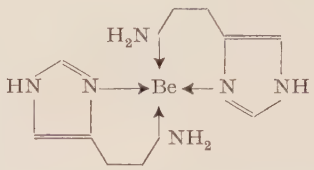

Fig. 4

In vivo, la vérification du blocage de l'histamine des nerfs a été faite avec le concours de C. Champy suivant la méthode décrite[1]. La poussière métallique mise au contact d'une sous-maxillaire de rat fait disparaître la réaction de histamine sur les terminaisons nerveuses renfermant l'amine, quand le témoin donne une coloration parfaite.

La fixation de l'histamine par les substances cancérigènes semble bien présenter un caractère de généralité.

Simone Hatem

Laboratoire du C.N.R.S., Faculté de Médecine de Paris, le 21 avril 1960.

Summary

In the case of mineral carcinogenic substances, the relation already established for organic carcinogenic substances with histamine is apparent. Silver, nickel, cobalt, chronium, and berryllium form well-characterized complexes with histamine.

Action des enzymes sur la gonadotropine urinaire de ménopause

De nombreux travaux rapportent l'action d'enzymes sur les hormones gonadotropes chorionique, sérique ou hypophysaire. Seule, la gonadotropine urinaire de ménopause semble ne pas avoir été l'objet d'une telle étude. Il est vrai que cette hormone est la moins bien caractérisée des gonadotropines. Cependant, ayant obtenu, à partir de l'urine de femme ménopausée, par une méthode de préparation originale [1, 2] une gonadotropine très purifiée, il nous a paru intéressant de suivre les effets que pourraient avoir les principaux enzymes sur son activité biologique.

[1] R. Bourrillon, R. Got et R. Marcy, Nature *184*, 983 (1959).
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Activité biologique de la Gonadotropine urinaire de ménopause soumise à l'action de différents enzymes

Conditions d'expérience	Durées d'incubation			
	0 h	1 h	6 h	24 h
Témoin	21 ± 3			
Témoin pH 7,3				24 ± 4,5
Témoin pH 6,3				21,5 ± 3
Témoin pH 8				19,5 ± 1,5
Témoin pH 5,5				28 ± 8
Témoin pH 2			10,5 ± 2,5	
Pepsine			10,5 ± 3	
Trypsine		26 ± 7	22 ± 4,5	10 ± 1,5
Chymotrypsine		23 ± 5	22 ± 4	8 ± 3,5
Papaïne		19 ± 3	18 ± 2,5	12 ± 4,5
Carboxypeptidase		9,5 ± 3,5		
Glucosidase		21 ± 4	24 ± 5	14 ± 4,5
RDE		10 ± 1,5		

L'activité est exprimée en poids d'utérus de souris pour un poids de 0,01 mg d'hormone injectée. Le poids des utérus des animaux non injectés est de 5,5 mg ± 1,5.

Méthodes. Le substrat était une préparation hormonale 100 fois plus active que le standard HMG 24.

Le test biologique était celui de l'augmentation du poids d'utérus chez la souris impubère de 21 jours et de 10–12 g ; une seule injection était pratiquée, et les animaux étaient sacrifiés et disséqués 72 h après l'injection[3]. Cinq souris étaient utilisées pour chaque échantillon à doser.

Les enzymes protéolytiques et la glucosidase étaient des produits purs cristallisés (Worthington). La neuraminidase était une préparation de «Receptor Destroying Enzyme» (Behringwerke). Chaque enzyme était utilisé à son pH optimum, en milieu tamponné: pH 7,3 pour trypsine, chymotrypsine et papaïne, pH 2 pour la pepsine, pH 8 pour la carboxypeptidase, pH 5,4 pour la glucosidase et pH 6,3 pour le RDE. Le rapport enzyme/substrat était 1/50. Les incubations étaient effectuées à 37° sous toluène. Pour chaque essai, une solution témoin d'hormone était préparée dans les mêmes conditions, mais sans enzymes.

Des quantités identiques (0,01 mg/par animal) d'hormone témoin et d'hormone ayant subi l'action des enzymes étaient injectées, au bout des temps d'incubation déterminée. Ainsi pouvaient être mis en évidence le maintien ou la perte d'activité, selon que les moyennes des poids d'utérus obtenus étaient égales ou présentaient une variation significative.

Résultats. Plusieurs séries d'expériences ont été effectuées pour chaque enzyme (350 souris ont été utilisées). Le tableau résume les résultats obtenus.

Le poids d'utérus des animaux non injectés est de 5,5 ± 1,5 mg. L'injection de 0,01 mg d'hormone non incubée donne un poids d'utérus de 21 ± 3 mg (moyenne de 50 animaux).

Remarquons tout d'abord qu'à pH 7,3, 8, 6,3 ou 5,4, l'incubation de l'hormone à 37°, durant 24 h (durée maximum des temps d'action des enzymes), n'a aucune action sur son activité, lorsque les enzymes sont absents. Au contraire, à pH 2, une chute importante est enregistrée au bout de 6 h; cette perte d'activité du témoin interdit toute étude de l'action de la pepsine.

La trypsine, la chymotrypsine, la papaïne semblent ne pas avoir d'action au bout de 6 h d'incubation, puisqu'aucune modification significative du poids d'utérus n'est enregistrée. D'ailleurs, les essais effectués pour des temps intermédiaires confirmaient ce résultat. L'incubation prolongée durant 24 h entraînait une perte sensible de l'activité; des essais supplémentaires ont permis, grâce à l'injection de concentrations plus élevées d'hormone incubée, de chiffrer cette perte à environ 50% en 24 h.

La carboxypeptidase a, au contraire, une action très rapide, puisqu'au bout d'une heure, on ne retrouve pratiquement plus d'augmentation du poids d'utérus, par rapport à l'animal non injecté.

La gonadotropine urinaire étant une glycoprotéine, comme toutes les gonadotropines, il était intéressant d'étudier également l'action de certains enzymes glucidolytiques. Ainsi, la glucosidase, sans action, comme les endopeptidases utilisées, durant 6 h, semble entraîner une légère perte d'activité au bout de 24 h.

Quant au RDE, son action, comme celle de la carboxypeptidase est très rapide, puisqu'au bout d'une heure, la perte d'activité est très nette. Ce résultat, lié à la libération des acides sialiques, est à rapprocher de celui obtenu par la seule incubation à pH 2: en effet, il est vraisemblable que la perte d'activité en milieu acide est due, au moins en partie, à la rupture de la liaison labile des acides sialiques avec la molécule.

En conclusion, les exopeptidases inactivent rapidement (1 h) cette hormone, tandis que les endopeptidases n'ont aucune action significative sur l'activité biologique pendant les six premières heures d'incubation. Ces résultats peuvent être opposés à ceux obtenus pour l'autre gonadotropine urinaire, l'hormone chorionique: en effet, cette gonadotropine perdait environ 60% de son activité après 6 h d'incubation par la trypsine, la chymotrypsine et la papaïne, alors qu'elle résistait 3 h à l'action de la carboxypeptidase[4].

R. Got et R. Bourrillon

Laboratoire de Chimie Médicale, Faculté de Médecine, Paris, le 13 juillet 1960.

Summary

The action of various proteolytic and glucidolytic enzymes on the biological potency of Human Menopausal Gonadotropin was studied. Trypsin, chymotrypsin, papaïn and glucosidase attack the gonadotropin only after 6 h of digestion. Carboxypeptidase and RDE produce a rapid inactivation.

[3] P. J. Claringbold et D. R. Lamond, J. Endocrinol *16*, 86 (1957).

[4] R. Bourrillon, R. Got et R. Marcy, Acta Endocrinol. *31*, 553 (1959).

In vitro Study of Fe-Cystine Chelate Implicated in Fusariose Wilt of Cotton

Cystine is reported to withhold iron from toxic principle (s) of host-parasite interaction, thus preventing toxin potentiation[1]. This mechanism of chelation, involving cystine, iron, and one of the phytotoxins (fusaric acid) implicated in cotton wilt has been investigated here *in vitro*.

I am most grateful to Professor T. S. Sadasivan for guidance and encouragement and to Dr. S. Suryanarayanan for valuable suggestions. I thank the University Grants Commission for the award of a Fellowship.

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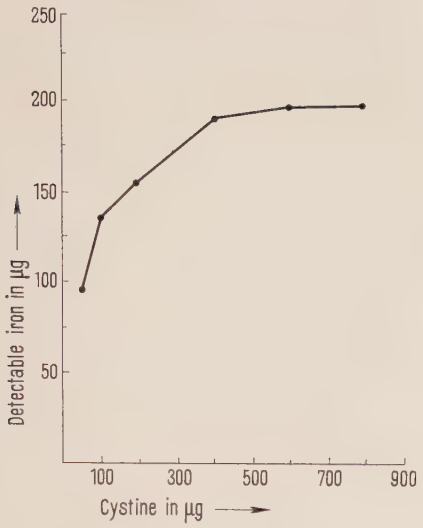


Fig. 1. Retention of iron in solution as effected by cystine.

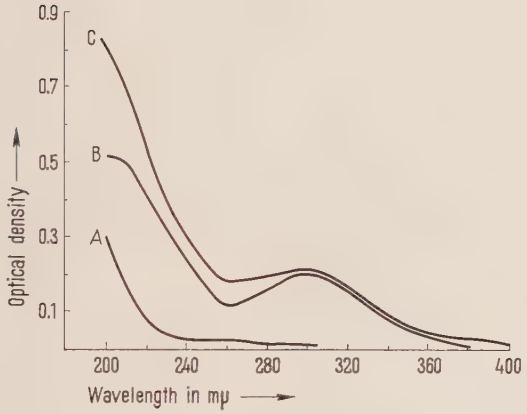


Fig. 2. Absorption spectra of A: cystine; B: iron; C: cystine-iron.

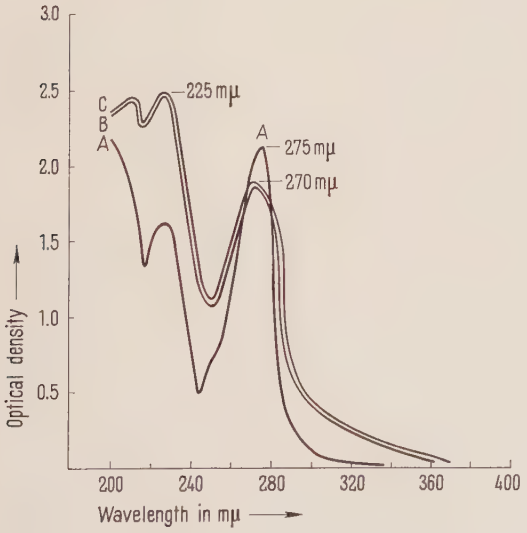


Fig. 3. Absorption spectra of A: fusaric acid; B: fusaric acid-iron; C: fusaric acid-cystine-iron.

R_f Values of Cystine, Iron, and Cystine-Iron

	Spray reagent used	No. of spots	Colour of spots	R_f	Area of spots in mm²
Iron	Potassium ferrocyanide	1	Blue	0.380	164
Cystine	Ninhydrin	1	Purple	0.210	194
Cystine-iron	Potassium ferrocyanide	1	Blue	0.389	169
Cystine-iron	Ninhydrin	1	Purple	0.210	196

Colorimetry: If cystine and iron chelate, it ought to affect precipitation of iron hydroxide from an aqueous solution of ferric iron, as metals bound in chelate rings lose their ionic characteristics and are less subject to participation in chemical reactions. Therefore, increasing concentrations of cystine (50–900 µg) were added to 200 µg aliquots of iron in aqueous ferric ammonium sulphate (pH 4–5), volume made up to 10 ml, left overnight, centrifuged, and supernatent analysed for iron spectrophotometrically in terms of intensity of colour with thioglycolic acid and a,a'-dipyridyl. Figure 1 indicates that the amount of iron detected in solution is directly proportional to the amount of cystine added.

Chromatography: Ascending chromatograms were run, using cystine, iron, and cystine-iron combinations (with *N*-butanol : HCl : water in the ratio 4:1:2, as solvent). The quantity of cystine and iron spotted being constant, the R_f and area of spots developed by cystine and iron individually, correspond to those given by cystine-iron combinations (Table).

Spectrophotometry as a technique for estimating chelates has been reported by Hill-Cottingham[2]. Absorption spectra of cystine, iron, and fusaric acid, and combinations of the three were studied. Results show that the spectral pattern of cystine-iron combination is merely an additive one of the individual spectra of the two substances (Figure 2). The spectrum of fusaric acid, however, is altered by the addition of iron, and this iron-fusaric acid spectrum remains uninfluenced by cystine (Figure 3).

Discussion: The foregoing observations seem to rule out the possibility of a chelation between cystine and iron *in vitro*. If chelation is involved, the consequent change in molecular configuration should result in an altered spectral pattern and, indeed, spectral patterns (Figures 2 and 3) show no such alteration indicative of chelation. Chromatographic findings also seem to confirm the spectrophotometric results. However, the retention of iron in solution in the presence of cystine (Figure 1) may be due to the surface activity of cystine, as similar instances are now well known[3]. If a labile hydrated complex is formed between cystine and iron, it evidently does not affect the availability of iron for other reactions.

The repercussions of the above findings in the context of *Fusarium* wilt of cotton seem far reaching. The applicability, however, of these *in vitro* findings to *in vivo* studies remains to be investigated.

S. Malini

University Botany Laboratory, Madras (India), June 27, 1960.

[2] D. G. Hill-Cottingham, Nature *175*, 347 (1955); Analyst *80*, 906 (1955).

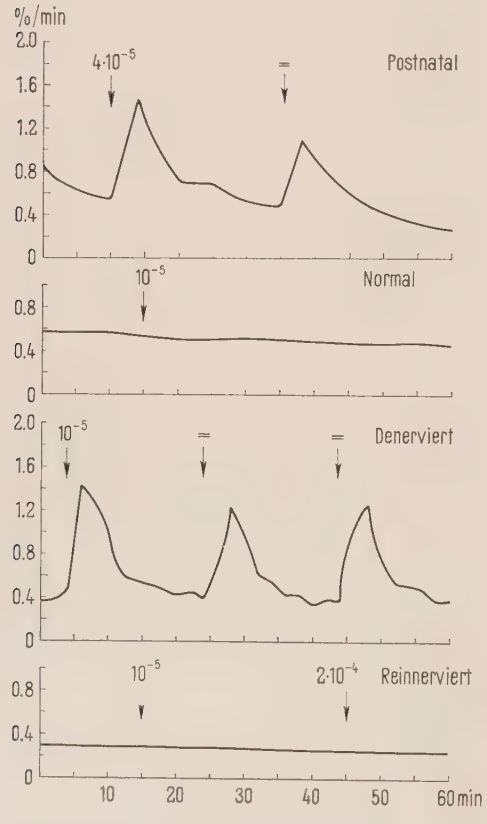
[3] M. Milone and G. Cetini, Ann. Chim., Rome *41*, 326 (1951). – W. A. Delong and M. Schnitzer, Soil Sci. Soc. Amer. Proc. *19*, 360 (1955).

Zusammenfassung

Die Möglichkeit einer Chelatbildung zwischen Cystin, Fusarinsäure und Eisen *in vitro* wurde untersucht. Während Fusarinsäure und Eisen einen stabilen Komplex bilden, ergaben kolorimetrische, chromatographische und spektrophotometrische Untersuchungen wenig Anhaltspunkte für eine Chelierung von Cystin und Eisen.

Der Einfluss von Acetylcholin auf die ^{42}Kalium-Abgabe postnataler, denervierter und reinnervierter Skeletmuskulatur

Sowohl das denervierte wie auch das embryonale bzw. postnatale Rattenzwerchfell sind überempfindlich gegenüber Acetylcholin (ACh). Dies lässt sich durch die Ausbildung einer ACh-Kontraktur (am denervierten Muskel Muscholl und Lüllmann[1], am embryonalen Muskel Rückert[2]) und durch Messung des Membranpotentials (Lüllmann und Pracht[3], Axelsson und Thesleff[4], Diamond und Miledi[5]) demonstrieren. Die Depolarisation des denervierten Skeletmuskels durch ACh wird durch eine Zunahme der Ionenpermeabilität verursacht, die unter anderem auch für Kaliumionen gilt (Klaus, Kuschinsky, Lüllmann und Muscholl[6]). Wir prüften jetzt, ob die Steigerung der ^{42}K-Abgabe durch ACh auch am postnatalen Skeletmuskel des Warmblüters auftritt und ob der reinnervierte Muskel sich wieder wie ein normalinnervierter Muskel verhält. Mit der an anderer Stelle ausführlich dargestellten Methode (Klaus, Lüllmann und Muscholl[7]) wurde die Aktivität des mit ^{42}K aufgeladenen Muskels und die von dem Muskel abgegebene ^{42}K-Menge fortlaufend registriert. Wir benutzten für unsere Versuche Zwerchfelle von Ratten am 3.–4. Tage *post natum*, Kontrollmuskeln von 100–120 g schweren Ratten, Zwerchfelle 8–12 Tage nach Exhärese des N. phrenicus und Muskeln, die nach etwa 20 Wochen reinnerviert waren.



Die ^{42}K-Abgabe (%/min) am postnatalen (4 Tage nach der Geburt), normalen, denervierten (9 Tage nach Denervation) und reinnervierten (21 Wochen nach Denervation) Rattenzwerchfell unter der Einwirkung von Acetylcholin. Die Acetylcholinkonzentrationen sind in g/ml angegeben.

Die Versuchsresultate sind jeweils durch ein Beispiel in der Abbildung dargestellt. Das postnatale Zwerchfell reagiert auf Zusatz von ACh mit einer Zunahme der ^{42}K-Abgabe. Der normale Muskel und das reinnervierte Zwerchfell sind unempfindlich gegenüber ACh, während der denervierte Skeletmuskel mit einer starken Zunahme der ^{42}K-Abgabe reagiert. Die ACh-Reaktion des Zwerchfells 3 und 4 Tage nach der Geburt ist schwächer als die eines 7–14 Tage lang denervierten Muskels unter gleichen Versuchsbedingungen. Wie Diamond und Miledi[5] zeigen konnten, sind zu diesem Zeitpunkt die endplattenfernen Muskelabschnitte bereits unempfindlich gegenüber ACh geworden. Somit wird nur ein Teil der Muskelzelloberfläche zur ACh-Reaktion beigetragen haben. Das reinnervierte Rattenzwerchfell verhält sich mit Ausnahme des histologischen Querschnittbildes wie ein normaler Muskel (Kuschinsky, Lüllmann, Hoefke und Muscholl[8]; Lüllmann und Pracht[3]). Es ist daher auch nicht erstaunlich, dass der reinnervierte Skeletmuskel keine Überempfindlichkeit gegenüber ACh besitzt. Die postnatale Ausreifung des Nervus phrenicus (Peters und Muir[9]) und der Reinnervationsprozess führen also zu demselben Ergebnis: Einengung des ACh-empfindlichen Areals auf die Endplattenregion.

W. Klaus, H. Lüllmann und E. Muscholl

Pharmakologisches Institut der Universität Mainz, 1. August 1960.

Summary

The loss of ^{42}K from the isolated rat diaphragm into the bathing solution is increased by acetylcholine, (a) 3 to 4 days after birth, (b) 7 to 14 days after sectioning of the phrenic nerve. The ^{42}K loss is not altered by acetylcholine in the diaphragm of adult rats and after the denervated diaphragm has become reinnervated.

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[2] W. Rückert, Arch. exp. Path. Pharmak. *150*, 221 (1930).
[3] H. Lüllmann, und W. Pracht, Exper. *13*, 288 (1957).
[4] J. Axelsson und S. Thesleff, J. Physiol. *147*, 178 (1959).
[5] J. Diamond und R. Miledi, J. Physiol. *149*, 50 P. (1959).
[6] W. Klaus, G. Kuschinsky, H. Lüllmann und E. Muscholl, Med. exp. *1*, 8 (1959).
[7] W. Klaus, H. Lüllmann und E. Muscholl, Pflüg. Arch. ges. Physiol. *271*, 761 (1960).
[8] G. Kuschinsky, H. Lüllmann, W. Hoefke und E. Muscholl, Anat. Anz. *103*, 116 (1956).
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Elektrolytgehalt von Endo- und Perilymphe in den einzelnen Schneckenwindungen des Meerschweinchens ohne und mit Stimulation

Natrium- und Kaliumbestimmungen in der Endolymphe beim Meerschweinchen[1], bei der Katze[2] und beim Menschen[3] ergaben einen sehr hohen Kalium- und einen geringen Natriumgehalt. Die Endolymphe hat also in bezug auf diese Elektrolyte einen intrazellulären Charakter, während die Perilymphe, die in ihrer Zusammensetzung dem Liquor cerebrospinalis gleicht, von extrazellulärem Typus ist.

Die üblichen Methoden der Gewinnung mittels Punktionen lassen zu viele Fehlerquellen offen, und einzelne Windungen konnten bis jetzt nicht getrennt untersucht werden. Mit einer Gefriermethode können die Schwierigkeiten der Punktion umgangen und zusätzlich chemische Abläufe zu einem ganz bestimmten Zeitpunkt untersucht werden. Der guillotinierte Meerschweinchenkopf, dessen Mittelohrräume durch einen Fräsenschnitt freigelegt werden, wird in flüssigem Stickstoff (– 180°C) kältefixiert. Da gefrorenes Wasser grösseren Raum beansprucht als in flüssigem Zustand, ist es notwendig, die Unterkühlung durch Eintauchen in Isopentan von zunehmendem Kältegrad während Sekundenbruchteilen einzuleiten. Im Kühlraum von –15°C wird die knöcherne Cochleakappe abgefräst; die gefrorenen Peri- und Endolymphräume werden dann unter dem Mikroskop präpariert und gesammelt. Wurde vorher Na-24 oder K-42 injiziert, um die Austauschgeschwindigkeit der radioaktiven Elektrolyte zu erfassen (300 μc/Meerschweinchen, das heisst etwa 0,6–0,7 μc/g Tiergewicht 4–6–12 h vor dem Tod in isotonischer Lösung intraperitoneal), konnten in dem gesammelten Material zuerst die Impulse/min gemessen und danach flammenphotometrisch der Gesamtgehalt an Natrium und Kalium bestimmt werden.

Versuchsanordnung. Serien von 15–20 Meerschweinchen, das heisst 30–40 Ohren, wurden a) ohne Tonreiz, b) nach Beschallung mit 2000 Hz und 140 db während 4 h – was praktisch einer Ertaubung entspricht – wie oben beschrieben getötet, präpariert und chemisch ausgewertet.

Ergebnisse. 1. Unbeschalltes Ohr

a) *Verhalten von Kalium.* Während die Perilymphe der Scala vestibuli in allen Windungen ungefähr gleich viel Kalium enthält (17 ± 2 meq/l), beträgt der Kaliumwert in der Basalwindung der Scala tympani nur die Hälfte bis ein Drittel, erreicht aber in der 2.–4. Windung wieder die Werte der Scala vestibuli.

Diese Unterschiede kommen auch mit dem markierten K-42 zum Ausdruck. Ausserdem scheint die Austauschmenge in beiden Perilymphräumen von der ersten zur zweiten Windung zuzunehmen.

Die Endolymphe enthält in allen Windungen ungefähr gleich viel Kalium; auch hier ist der K-42-Austausch in der zweiten Windung lebhafter als in der ersten.

b) *Verhalten von Natrium.* Die Natriumwerte der Scala vestibuli entsprechen denjenigen der Scala tympani in derselben Höhe. Zwischen den einzelnen Windungen besteht allerdings ein Unterschied, indem die Werte von basal nach apikal deutlich abnehmen, so dass zum Beispiel in der dritten Windung der Scala vestibuli der Natriumgehalt nur noch zwei Drittel beträgt (70 meq/l) im Vergleich zur Basalwindung (105 meq/l).

Die Austauschwerte an Na-24 lassen weder von einer Windung zur anderen noch zwischen den beiden Perilymphräumen einen Unterschied erkennen.

Der Natriumgehalt der Endolymphe scheint in allen Windungen unverändert zu sein (58 meq/l), während die Na-24-Werte von basal nach apikal deutlich zunehmen.

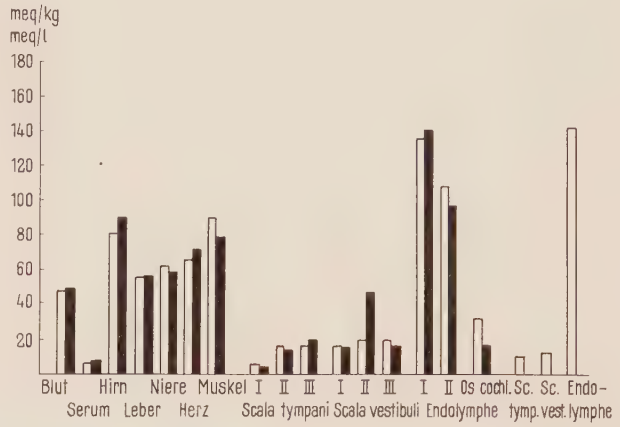

Fig. 1. Kaliumverteilung in Peri- und Endolymphe einzelner Windungen sowie in einigen Organen ohne und mit Stimulation von 2000 Hz, 140 db während 5 h.

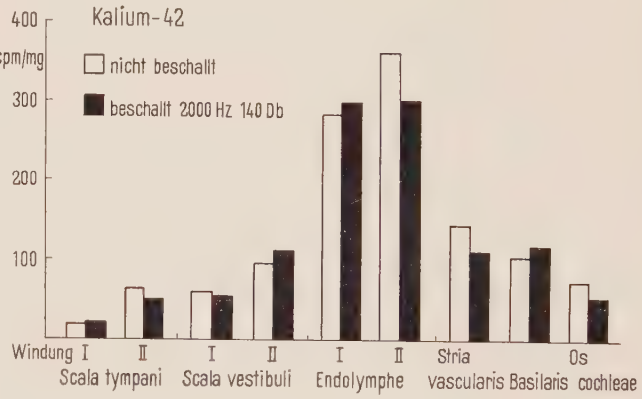

Fig. 2. Verteilung von K-42 in Peri- und Endolymphe 5 h nach intraperitonealer Injektion von K-42 ohne und mit Stimulation von 2000 Hz, 140 db während 5 h. Die Isotopenmessung und Berechnung führte Dr. R. A. Collet, Isotopenlabor, Zentrallaboratorium des Kantonsspitals Genf, durch.

Die Arbeiten wurden mit Unterstützung des Schweizerischen Nationalen Forschungsfonds durchgeführt.

Technische Hilfe leisteten uns in dankenswerter Weise Dr. R. A. Collet, Isotopenlabor, Zentrallaboratorium des Kantonsspitals, und Dr. Fischer (Kältekammer von – 15°C), Centre de transfusion, Kantonsspital Genf.

[1] C. A. Smith, O. H. Lowry and M. L. Wu, Laryngoscope *64*, 141 (1954).

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[3] S. Rauch und A. Köstlin, Pract. Oto-rhino-laryng. *20*, 287 (1958).

[4] Gegenwärtige Adresse: Department of Biochemistry, University of Oxford (England).

2. Nach Beschallung mit 2000 Hz und 140 db während 4–6 h

a) *Verhalten von Kalium.* Am auffallendsten ist der ungefähr dreifache Anstieg des Kaliumgehalts in der zweiten Windung der vestibulären Perilymphe, das heisst in Höhe der Tonperzeption, während die Kaliumwerte weder in der ersten, dritten und vierten vestibulären Windung noch in einer der tympanalen Windungen nennenswerte Änderungen erfahren.

Dieser auffallende Unterschied kommt mit K-42 nicht zur Darstellung.

Der totale Kaliumgehalt der Endolymphe wie der K-42-Austauschwert nehmen in der zweiten Windung leicht ab (15–20%), während in den übrigen Windungen keine Veränderungen zu erkennen sind.

b) *Verhalten von Natrium.* Der Natriumgehalt nimmt in beiden Perilymphräumen nach Beschallung leicht zu, apikal stärker als basal. Wesentlich ausgesprochener sieht man diese Zunahme in den Na-24-Austauschwerten, und zwar sowohl in der Peri- wie in der Endolymphe (durchschnittlich um 15% in der 1. Windung, um 20–40% in der 2. Windung, 40–60% in der 3. und über 60% in der 4. Windung).

Die übrigen Partien der Cochlea, die Basalmembran und die knöcherne Kappe zeigen gleichgerichtete Veränderungen mit Ausnahme der Stria vascularis, deren Na-24-Austauschwerte selbst abnehmen.

Folgerungen. Trotz der Diffusionsmöglichkeiten ergeben sich erstaunlich grosse Unterschiede einerseits zwischen der tympanalen und vestibulären Perilymphe, andererseits zwischen den verschiedenen Windungen. Dieser Befund, erhoben im Ruhezustand, wird nach Einton-Reizung noch augenfälliger.

Da nach histologischen Befunden Töne von 2000 Hz in der zweiten Windung perzeptiert werden, finden die chemischen Veränderungen in der zweiten Windung nach Ertaubung eine Erklärung.

Ausserdem scheinen sowohl die höheren Kaliumwerte in der vestibulären Perilymphe, die in der Basalwindung besonders hervortreten, wie auch ihr deutlich erhöhter Kaliumgehalt in der zweiten Windung nach Beschallung ein Hinweis zu sein, dass die Reissnersche Membran zu gegebener Zeit zum mindesten partiell semipermeabel ist.

Weit interessanter wird es sein, den Effekt zu verfolgen, den ein Einzelton von verschiedener Intensität an chemischen Veränderungen in der Peri- und in der Endolymphe auslöst.

S. Rauch[4]

Zentrallaboratorium des Kantonsspitals Genf (Schweiz), 30. Mai 1960.

Summary

A freezing method is described which permits the measurement of electrolytes and enzymes in the peri- and endolymph from a single turn of the cochlea of guinea pig at a known time. The perilymphs of the scala vestibuli and the scala tympani are different, especially in the first basic turn. This difference may be produced by a partial semipermeability of the Reissner's membrane. After producing deafness by a sound of frequency 2000 c/s, the potassium (the total potassium level detected chemically and by the use of potassium-42) increases in the perilymph of the second turn only, and decreases slightly in the endolymph of the same turn. In the other turns, the potassium does not change.

This method permits the analysis of chemical changes due to the stimulus of single sounds.

The Effect of Methyl Alcohol on the Conversion of Thiamine to Thiochrome[1]

Oxidation products of thiamine have been studied extensively by Barger *et al*[2], Jansen,[3] Zima and Williams[4], Sykes and Todd[5], and Mizuhara and Arata[6]. The major product of alkaline oxidation of thiamine by $K_3Fe(CN)_6$ is thiochrome. Jansen[3] using the thiamine-thiochrome conversion as a quantitative determination of thiamine, suggested the addition of CH_3OH to the oxidation mixture to prevent «harmful» action of the excess ferricyanide. Later investigators apparently omitted CH_3OH but obtained only a 65–67% conversion[7–9]. Since no reliable data (except Jansen's work in 1936) on the influence of CH_3OH on the thiamine-thiochrome conversion seemed to be available, studies were made to determine the effect. The data indicate that addition of 25% (vol/vol) CH_3OH to the alkaline oxidation mixture results in better than 90% conversion in the concentration range usually employed.

Procedures. Standard stock solutions contained 100 mg% thiamine hydrochloride (Merck and Company) or 100 mg% thiochrome (Nutritional Biochemicals) in 0·1N HCl. These were diluted with 0·1N HCl to give 0·1, 0·2, 0·4, 1·0, 200 and 1000 µg/ml of solution.

To 1 ml samples of the diluted 0·1N HCl thiamine solutions were added 0 to 3 ml of redistilled CH_3OH, 3 ml of 0·3% $K_3Fe(CN)_6$ in 15% NaOH and distilled water to make a total of 8 ml of solution. The same reagents were added to the thiochrome solutions. 15 ml of redistilled isobutanol were then added and the mixtures shaken vigorously for $1^1/_2$ min. Blanks were run by omitting $K_3Fe(CN)_6$ from the reaction mixture. After centrifuging at 500–600 r.p.m. for 45 sec, 10 ml of the top isobutanol layer (or an appropriate dilution of this layer with isobutanol) was pipetted into a 19 × 105 mm cuvette and the fluorescence measured in a Coleman photofluorometer using 0·01 mg% quinine sulfate in 0·1N H_2SO_4 as a reference standard. Thus the fluorescence of thiochrome derived from a known amount of thiamine in the reaction mixture was compared to the fluorescence of a comparable amount of thiochrome treated with the same reaction mixture. To investigate the validity of this procedure, fluorometric readings of thiochrome solutions were compared as follows:

(a) Thiochrome dissolved in the reaction mixture as described above, containing 0 and 2 ml respectively of CH_3OH and extracted into isobutanol by shaking for $1^1/_2$ min.

(b) Thiochrome dissolved directly in isobutanol.

The results of this series are given in Table I. The data indicate a somewhat lower recovery of thiochrome when CH_3OH is omitted from the reaction mixture. However,

[1] This research was supported specifically by the Nutrition Foundation Inc. (Grant No. 257), also generally by Office of Naval Research and the Army Surgeon General (Contract NONR-1623), by National Institutes of Health (Grant A-566) and by the University of Notre Dame.

[2] G. F. Barger, F. Bergel, and A. R. Todd, Ber. dtsch. chem. Ges. *68*, 2257 (1935).

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[9] J. W. Ferrebee and G. A. Carden, J. lab. clin. Med. *25*, 1320 (1940).

Tab. I. Fluorometer Readings for 0.3 μg Thiochrome Using Quinine Standard Setting of 50 (see text). Average Readings of 6 Series of Experiments. SDM Values Given.

	Thiochrome in 8 ml reaction mixture + 15 ml isobutanol		Thiochrome in 15 ml isobutanol
Ml CH_3OH in reaction mixture	2	0	—
Final volume of isobutanol phase (ml)	17.6	16.0	15.0
Average reading corrected to volume of 15 ml isobutanol phase	63.4 ± 0.8	58.1 ± 1.4	63.1 ± 0.6

Tab. II. Percentile Conversion of Thiamine Hydrochloride to Thiochrome in an Alkaline Ferricyanide Solution Using Various Amounts of Methyl Alcohol in a Total Reaction Mixture of 8 ml. All Data Are the Average of Three or More Determinations.

ml CH_3OH	μg Thiamine Hydrochloride					
	0.1	0.2	0.4	1.0	200	1000
0	65.2	67.1	69.6	69.1	60.6	65.2
1	90.7	89.2	85.3	81.3	78.6	77.5
2	92.3	94.4	94.0	87.8	84.0	80.3
3	93.1	97.1	96.1	86.7	79.3	75.4

the effect is small and possibly can be ascribed to incomplete extraction into the isobutanol phase. In the presence of CH_3OH, the recovery of thiochrome from the reaction mixture is complete.

Results and Discussion. Theoretically, assuming 100% conversion of thiamine to thiochrome, 1 μg of thiamine hydrochloride should produce 0·778 μg of thiochrome. Preliminary series containing 0·1 to 0·4 μg of thiamine and thiochrome respectively were run using 2 ml of CH_3OH in the reaction mixture. In this low concentration range the yield of thiochrome was not 100%, but a conversion of over 90% was indicated. Optimal conditions for maximum conversion were then determined by varying the amount of CH_3OH from 0 to 3 ml and also the range of thiamine concentration was increased. The results are given in Table II.

These data indicate that in both the low concentration range (0·1 to 1·0 μg/8 ml of reaction mixture) and the higher concentration range (200 and 1000 μg/8 ml reaction mixture) there is a general increase in the percent conversion when the amount of CH_3OH is increased from 0 to 2 ml. In the lower range (using 2 ml of CH_3OH) the average maximum conversion is greater than 90% confirming Jansen's[3] original results, while in the higher range the average conversion is still better than 80%. When CH_3OH is omitted from the reaction mixture there ist a 60–70% conversion in both the high and low ranges, confirming the results of Connor and Straub[7], Egana and Meiklejohn[8], and Ferrebee and Carden[9].

As Zima and Williams[4] pointed out, thiamine exists in solution in a state of equilibrium between the ammonium and the thiol type molecule. The equilibrium shifts to the ammonium type in an acid medium, while alkaline solutions favor the thiol type. Alkaline oxidation as used in this procedure could result in two types of reactions:

(a) An oxidative conversion of thiamine to thiochrome (Sykes and Todd[5]).

(b) An oxidative dimerization of the thiamine molecule to a non-fluorescent disulfide type molecule. Subsequently thiaminedisulfide could, under the conditions provided by the reaction mixture, possibly decompose to yield additional thiochrome (Zima and Williams)[4] and presumably thiamine thiazolone (Sykes and Todd)[5].

The data could be interpreted to indicate that in the absence of CH_3OH, part of the thiamine would follow the bimolecular type reaction. The presence of CH_3OH in the alkaline reaction mixture would change conditions to such an extent that more of the thiamine follows a monomolecular reaction type and is converted directly to thiochrome. Thus in the lower concentration range, the presence of CH_3OH (25% vol/vol) results in over 90% of the thiamine being oxidized to thiochrome according to the first reaction type. At higher concentrations, which would favor a bimolecular type reaction, the second reaction seems to become increasingly important.

B. S. Wostmann and P. L. Knight

Lobund, Department of Biology, University of Notre Dame and St. Mary's College, Notre Dame (Indiana), July 5, 1960.

Zusammenfassung

Der Einfluss verschiedener Methylalkoholkonzentrationen auf die Thiamin-Thiochrom-Umsetzung im alkalischen Milieu mit 0·11% $K_3Fe(CN)_6$ wurde verfolgt. Im Reaktionsgemisch ohne CH_3OH wird im untersuchten Konzentrationsbereich (0·1 bis 1000 μg in 8 ml Reaktionsgemisch) ungefähr 65% des Thiamins in Thiochrom umgewandelt. Im Reaktionsgemisch mit 2 ml CH_3OH in 8 ml wird der Umsatz über 90% für Thiaminkonzentrationen unter 1 μg und über 80% im Konzentrationsbereich von 1 bis 1000 μg Thiamin in 8 ml Reaktionsgemisch gesteigert.

Occurrence of Soluble Pigments in the Genus *Bacillus*

Pigmentation among species of the bacterial genus *Bacillus* is reported to be rare[1]. A few members of the genus do form pigmented colonies when cultured on agar media under certain conditions[2]. Little information has been published relative to the production of soluble pigments by the organisms grown in broth cultures. The above references do not describe pigmentation in broth by any members of the genus. However, *Bacillus anthracis* has been reported to produce a red[3] or purple-brown pigment[4] when cultured in liquid media. The present investigation was carried out to determine to what extent the common members of the genus *Bacillus* produced soluble pigments in broth cultures.

This investigation was supported by research grant E-1535 from the National Institutes of Health, U. S. Public Health Service.

[1] R. S. Breed, E. G. D. Murray, and N. R. Smith, *Bergey's Manual of Determinative Bacteriology*, 7th Ed. (Williams and Wilkins, Baltimore 1957), p. 613.

[2] G. S. Wilson and A. A. Miles, *Topley and Wilson's Principles of Bacteriology and Immunity*, 4th Ed. (Williams and Wilkins, Baltimore 1955), p. 952.

[3] G. G. Wright, M. A. Hedberg, and J. B. Slein, J. Immunol. *72*, 263 (1954).

[4] R. E. Strange and F. C. Belton, Brit. J. exp. Path. *35*, 153 (1954).

The cultures employed were obtained from the collection of Dr. K. L. Burdon of this department. Species identification of the organisms had been carried out by accepted methods[5]. The bacteria were cultured in the chemically defined, liquid medium of Thorne *et al.*[6] containing 0·7% sodium bicarbonate. Inoculations were made from vegetative cell suspensions into 25 ml of medium contained in 250 ml flasks. The cultures were tightly closed with rubber stoppers, and incubated at 37°C for 24 h on a rotary shaker.

For visual determination of soluble pigment production, cells were removed from the cultures by centrifugation. The clear supernatant broth then was adjusted to pH 7·5 with 0·1 N NaOH, and compared to a blank of uninoculated broth treated in the same fashion. Acid sensitivity of the pigments was determined by adding 1 N HCl to the broth. Fluorescence was detected by examining the culture supernates under an ultraviolet lamp emitting its maximum light at 2,750 Å. In certain cases the absorption spectra of the pigments were determined in a spectrophotometer.

As can be seen from the Table cultures belonging in two of the six species examined (*B. anthracis* and *B. cereus*) produced visible pigments. The pigment of *B. cereus* was associated with fluorescence of the culture filtrate, while that of *B. anthracis* was sensitive to acid. The characteristic pink pigment of the latter organism was evident only under alkaline conditions; the culture filtrate became yellow when acidified. Spectrophotometric examination in the visible range demonstrated a broad absorption spectrum for the anthrax pigment with a single peak at 490 mμ. The yellow-brown pigment of *B. cereus* had no characteristic absorption spectrum in the visible range, but in the ultraviolet region the material evidenced a single peak at 350 mμ. The pigment was not sensitive to acid.

Since preliminary investigations showed that *B. anthracis* and *B. cereus* produced soluble pigments, several strains of these species were examined to determine whether the phenomena were characteristic of the species. The anthrax specimens consisted of both virulent and avirulent organisms. The Table shows that 15 of the 20 strains examined produced pigment. All of the *B. cereus* strains employed demostrated both the yellow-brown pigment and fluorescence.

Johnstone and Fishbein[7] have suggested that florescent pigments might be used to distinguish species of *Azobacter*, and Brisou[8] has made important use of pigmentation in the classification of chromogenic Gram negative bacteria, including the genera *Achromobacter*, *Flavobacterium*, *Pseudomonas*, *Serratia*, and *Xanthomonas*. Our data indicate that the absence or occurrence of soluble pigments in broth cultures of *Bacillus* species might be employed as an adjunct to other available methods for their classification. Of the six species examined *B. anthracis* was the only one producing a pH-sensitive, pink pigment, and *B. cereus* was the only one in which a yellow-brown pigment and fluorescence occurred. However, since not all anthrax strains produced the pigment, the character could be useful only in those strains in which it appeared. Absence of pigmentation would not mean the organism was not *B. anthracis*. In addition it should be emphasized that the pigmentation reported occurs only under the growth conditions specified. Since many factors such as incubation temperature, media composition, pH, *etc.* can affect pigmentation of bacteria, we believe the characteristic should be employed only as an ancillary criterion in classification. As a case in point it should be mentioned that although the presence of cellular pigments is one of the important criteria for classification of the genus *Serratia*[1, 2], colorless strains have been reported[9] as well as one strain which produced a soluble pigment[10].

Soluble Pigment Production in Broth Cultures by *Bacillus* Species

Species	No. of Strains	Pigment	% Positive	pH Sensitivity of Pigment	Fluorescence
B. anthracis	20	Pink	75	Yes	No
B. cereus	18	Yellow-Brown	100	No	Yes
B. licheniformis	2	None	0		No
B. megaterium	2	None	0		No
B. pumilis	2	None	0		No
B. subtilis	3	None	0		No

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Department of Microbiology, Baylor University College of Medicine, Houston (Texas), April 4, 1960.

Résumé

B. anthracis cultivé en bouillon dans certaines conditions précisées dans ce travail, produit un pigment rose sensible aux variations de pH. *B. cereus* produit de même, un pigment jaune-brun, fluorescent. Les pigments sont solubles et se retrouvent dans le filtrat. Quatre autres espèces de *Bacillus* ne produisent pas de pigments dans les mêmes conditions. La production de pigments en bouillons de culture pourrait être employée comme un critère additionnel pour la classification des souches *Bacillus*.

[5] K. L. Burdon, J. Bacteriol. *71*, 25 (1956).
[6] C. B. Thorne, C. G. Gomez, and R. D. Housewright, J. Bacteriol. *63*, 363 (1952).
[7] D. B. Johnstone and J. R. Fishbein, J. gen. Microbiol. *14*, 330 (1956).
[8] J. Brisou, Ann. Inst. Pasteur *93*, 397 (1957).
[9] B. R. Davis, W. Ewing, and R. W. Reavis, Int. Bull. Bact. Nomen. Taxon. *7*, 151 (1957).
[10] R. P. Williams, W. W. Taylor, D. Hawkins, Jr., and I. L. Roth, Nature *182*, 1028 (1958).

Enzymatic Conversion of Metanephrine to Normetanephrine

Metanephrine (3-*O*-methylepinephrine) has been shown to be a major metabolic product of epinephrine and to occur normally in tissue and urine[1]. Since many methylated amines have been found to *N*-demethylate *in vitro*[2], the possibility that metanephrine might undergo such a reaction was examined.

[1] J. Axelrod, Physiol. Rev. *39*, 751 (1959).
[2] J. Axelrod, Arch. exp. Path Pharmak. *238*, 24 (1960).
[3] J. Axelrod, J. Pharm. exp. Therap. *114*, 430 (1956).
[4] J. Cochin and J. Axelrod, J. Pharm. exp. Therap. *125*, 105 (1959).
[5] J. Axelrod, S. Senoh, and B. Witkop, J. biol. Chem. *233*, 697 (1958).

Enzymatic N-Demethylation of Metanephrine

Microsomes obtained from 500 mg rabbit liver were incubated in air at 37°C with 3 μmoles metanephrine, 25 μmoles $MgCl_2$, 50 μmoles nicotinamide 50 μmoles neutralized semicarbazide hydrochloride, 0.25 ml pH 7.4 phosphate buffer (0.5 *M*), added cofactors and water to make a final volume of 3 ml. After 2 h, the incubated mixture was assayed for formaldehyde[4]

Additions	Formaldehyde Formed
	μmoles
Soluble fraction[a], TPN (0.5 μmole)	0.65
Soluble fraction[a]	0.20
Soluble fraction[a], TPN (0.5 μmole), microsomes omitted	0.00
TPN (0.5 μmole)	0.00
TPNH[b] (3 μmoles)	0.45
DPNH[b] (3 μmoles)	0.05

[a] Soluble fraction from 500 mg rabbit liver was dialyzed 20 h at 4°C against 0.01 *M* phosphate buffer pH 7.0.
[b] The cofactors were added in 6 divided portions over 90 min.

The enzymatic *N*-demethylation of metanephrine was studied by measuring the formaldehyde liberated after incubation of this amine with various cellular fractions of rabbit liver[3]. Incubation of microsomes with the soluble fraction of liver and triphosphopyridine nucleotide (TPN) resulted in the formation of formaldehyde (Table). When either the soluble fraction, TPN, or microsomes were omitted, little or no formaldehyde was formed. The soluble fraction and TPN could be partially replaced by reduced triphosphopyridine nucleotide (TPNH) but not reduced diphosphyopyridine nucleotide (DPNH).

Evidence for the identity of the *N*-demethylated product was obtained as follows: Microsomes and soluble supernatant fraction obtained from 5 g of rabbit liver were incubated at 37° with TPN, $MgCl_2$ and nicotinamide in pH 7·4 phosphate buffer. After 1 h the incubation mixture was adjusted to pH 9·5 and extracted twice with 5 vol of isoamyl alcohol. The isoamyl alcohol was re-extracted with 0·1N HCl and the acid extract was evaporated to dryness *in vacuo*. After taking up the residue in a small volume of ethanol, it was chromatographed on Whatman No. 1 paper, using isopropanol: ammonia (5%) 8:2 as the solvent system. The chromatogram was dried and a strip of an area corresponding to R_f 0·45 to 0·6 was cut out and eluted with methanol. The resulting extract contained a compound that gave the same R_f values in 3 solvent systems, color reactions and fluorescence spectrum as authentic normetanephrine[5]. These observations were taken as evidence that rabbit liver microsomes contained an enzyme requiring TPNH and other cofactors in the soluble fraction that could *N*-demethylate metanephrine to yield normetanephrine and formaldehyde. Guinea pig and rat liver also contain the metanephrine *N*-demethylating enzyme, but in smaller amounts.

The ability of metanephrine to form normetanephrine *in vivo* was examined after the administration of methoxy C^{14} metanephrine or H^3epinephrine to rats. No evidence for the presence of free and conjugated normetanephrine in the urine was found after the administration of these compounds.

J. Axelrod

Laboratory of Clinical Science, National Institute of Mental Health, Bethesda (Maryland), June 10, 1960.

Zusammenfassung

In der Mikrosomen-Fraktion der Leber von Kaninchen, Ratte und Meerschweinchen wurde ein Ferment gefunden, welches Metanephrin zu Normetanephrin unter Formaldehydbildung N-demethylieren kann.

Nach Verabreichung von markiertem Epinephrin und Metanephrin konnten bei der Ratte keine Anhaltspunkte für die Ausscheidung von Normetanephrin gefunden werden.

Elevated α-2-Serum Proteins as a Possible Genetic Marker in Spontaneous Hereditary Diabetes mellitus of the Chinese Hamster (Cricetulus griseus)[1]

The occurrence of a spontaneous hereditary diabetes mellitus in the Chinese hamster has recently been reported[2]. This is a primary pancreatogenic condition, with degranulation, hydropic degeneration, and deficiency of β-cells. Degenerative changes in β-cells associated with development of clinical diabetes occur at an age specific for each subline of diabetic hamsters. The incidence of diabetes among progeny from diabetic parents depends upon the stage of inbreeding, diabetes occurring in animals which are 80% or more homozygous.

Electrophoretic patterns of serum proteins in families with a high incidence of spontaneous diabetes reveal α-2 levels to be two to three times the normal values (Table). In normal animals, the α-2 proteins generally are between 5–10% of the total serum proteins. When high incidence families were randomly hybridized by single or double crosses involving two or four grandparents, respectively, of diabetic background, normal values of α-2 are re-established.

Ejarque *et al*[3] have summarized previous work and have also reported slightly elevated α-2 serum proteins, and protein-bound carbohydrates in patients with diabetes. This elevation of α-2 values occurred generally when the diabetes was complicated by vascular and other secondary changes. In the Chinese hamster, however, α-2 serum proteins are increased, even prior to the onset of clinical

Percentage of the Total Serum Proteins in the α-2 Fractions in Various Families of the Chinese Hamster and Hybrids

% Protein α-2 Fraction	Families					Hybrids	
	JFY	VSY	BUY	HGY	ORY	(1)	(2)
0 5	1	0	0	0	1	7	0
6–10	1	0	6	4	3	12	8
11–15	3	6	5	0	1	0	0
16 20	2	2	7	0	0	1	4
21–25	1	2	8	2	0	1	1
26–30	0	0	6	1	0	0	0
31 35	0	0	1	0	0	0	0
Total	8	10	33	7	5	21	13

[1] This investigation was supported in part by research grants from the National Institutes of Health, USPHS #E1560 and #CY3335, the National Science Foundation (#6-9602), and the Damon Runyon Memorial Fund (#293).

[2] H. Meier and G. Yerganian, Proc. Soc. exp. Biol. Med. *100*, 810 (1959).

[3] P. Ejarque, A. Marble, and E. F. Tuller, Amer. J. Med. *27*, 221 (1959).

[4] Present address: Jackson Memorial Laboratory, Bar Harbor, Maine (USA).

diabetes, and microscopic evidence of intercapillary glomerulosclerosis. Breeding experiments are now in progress, using elevated α-2 serum proteins as a genetic marker in an attempt to increase the incidence of spontaneous diabetes. The extremely high levels of α-2 (up to 35%) observed in diabetic hamsters may be due either to an increase of normally existing α-2 proteins, or perhaps to new and different molecules which are a genetic reflection of the diabetic state. Chemical analysis of the α-2 globulin fractions in diabetic and non-diabetic hamsters would be required in order to distinguish between these two possibilities; such investigations are now in progress.

M. N. Green, G. Yerganian, and H. Meier[4]

The Children's Cancer Research Foundation and the Department of Pathology, Harvard Medical School, at The Children's Hospital Medical Center, Boston (Massachusetts) May 10, 1960.

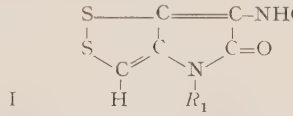
Zusammenfassung

Bei chinesischen Hamstern aus Inzuchten mit erblicher Zuckerkrankheit wurden erhöhte Serum-α-2-Eiweisswerte konstatiert. Die Erhöhung erfolgt vor Auftreten der Hyperglykämie oder Blutgefäss- und anderen sekundären pathologischen Veränderungen. Es wird gefolgert, dass die Eiweisserhöhung gen-bedingt ist und wahrscheinlich chemisch abnormales Eiweiss betrifft.

Isolation of *iso*-Butyropyrrothine along with Thiolutin and Aureothricin from a *Streptomyces* sp.

A new species of Streptomyces (*S. pimprina*) has been found to produce an undescribed antifungal antibiotic Hamycin of the polyene (heptaene) type[1]. Hamycin is present in the mycelium and the filtered broth was found to contain four antibiotics with antibacterial activity and, in addition, one colourless crystalline inactive compound.

The chloroform extract of the broth, on crystallization from benzene-methanol (60:40), gave a yellow crystalline compound (yield 50 mg/l), which was identified as the antibiotic Thiolutin[2] (I; $R = CH_3$; $R_1 = CH_3$) by comparison with an authentic sample (kindly supplied by Dr. A. C. Finlay of Chas Pfizer & Co., Brooklyn, N. Y.).

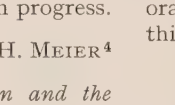


I

The mother liquor was chromatographed on paper, using benzene saturated with formamide as the developing phase, the paper having been soaked previously in acetone containing 20% formamide[3]. With ascending technique four yellow spots with the R_f (A) 0.0, (B) 0.4, (C) 0.71, and (D) 0.92 were obtained, which, when tested bioautographically with *Sarcina lutea* as the test organism, were found to be active.

The separation and the isolation of the antibiotics were done on cellulose powder, using formamide as the stationary phase and benzene saturated with formamide as the developing phase. Three moving bands together with one dark stationary band were observed. The three moving bands were collected separately and rechromatographed individually using a narrower column. The homogenity of each fraction was ascertained by paper chromatography.

The fast moving band (D, $R_f = 0.92$) was found to contain two components. One of them was an inactive colourless crystalline basic compound (D 1) which could be separated from the other by extracting a benzene solution of the mixture by dilute acid. Free base (yield, 20 mg/l) was crystallized from benzene into colourless prisms, m.p. 205°C [found, C 73.0, H 4.7, N 13.5%, M.W. 198 (Rast method)]. The other component was an orange-red active compound (D 2) which could be crystallized from benzene into orange-red plates, m. p. 228–229°C (found, C 47.4, H 4.8, N 10.9%) yield, 1 mg/l. This orange-red compound was identified as *iso*-butyropyrrothine[4]



(I; $R = -CH(CH_3)_2$; $R_1 = CH_3$)

from its analysis and its identical IR, and UV spectra, identical antimicrobial spectrum and undepressed melting point with a 'synthetic' *iso*-butyropyrrothine prepared by the action of *iso*-butyrylchloride on pyrrothine hydrochloride[2] in pyridine. The 'synthetic' *iso*-butyropyrrothine, when purified through cellulose column gave m.p. 228–229°C. Celmer and Solomons[5] mention its melting point as 222–228°C.

Natural *iso*-butyropyrrothine has an antimicrobial spectrum similar to Thiolutin. The *in vivo* inhibition concentration (in μg/ml) for various organisms is *Bacillus subtilis*, 1.25; *Sarcina lutea*, 0.45; *Staphylococcus aureus*, 1.0; *Candida albicans*, *Escherichia coli*, and *Salmonella paratyphi* were insensitive.

Another band (C, $R_f = 0.71$) gave a yellow-orange crystalline compound (yield 1 mg/l) which was identical to Aureothricin[6] (I; $R = CH_2{-}CH_3$; $R = CH_3$) in all obvious respects, including infra red, U. V. spectra, paper chromatographic behaviour and antimicrobial spectrum. 'Synthetic' Aureothricin was prepared by the method of Celmer and Solomons[2] from pyrrothine hydrochloride and propionic anhydride.

The third moving band (B, $R_f = 0.4$) was found to be Thiolutin itself.

The stationary band (A, $R_f = 0.0$) at the top of the column was removed and extracted with methanol and the methanol extract was chromatographed on alumina (Activity III) in dry chloroform. A band which was eluted with chloroform-methanol (99:1) showed activity against *S. lutea* but the UV absorption spectrum was different from either Thiolutin group or Holomycin[3] (I; $R = CH_3$, $R_1 = H$). This fraction (A) was obtained in very small quantity and is not characterised as yet.

Akabori and Nakamura[7] have obtained Thiolutin and Aureothricin along with 1-6-dihydroxyphenazine from a *Streptomyces* sp. To our knowledge, this is the first time that a *Streptomyces* sp. has been found to produce Thiolutin (acetopyrrothine), Aureothricin (propiopyrrothine),

Our thanks are due to Dr. M. J. Thirumalachar for his interest in this work.

[1] Hindustan Antibiotics Limited, Indian Patent Appl. No. 71394.

[2] W. D. Celmer and I. A. Solomons, J. Amer. chem. Soc. *77*, 2861 (1955).

[3] L. Ettlinger, E. Gäumann, R. Hütter, W. Keller-Schierlein, F. Kradolfer, L. Neipp, V. Prelog, and H. Zahner, Helv. chim. Acta *42*, 563 (1959).

[4] For the nomenclature see foot note 5a of [2].

[5] W. D. Celmer and I. A. Solomons, Antibiot. Ann. *1953–1954*, 622.

[6] H. Nishimura, T. Kimura, and M. Kuroya, J. Antibiot., Tokyo [A] *6*, 57 (1953).

[7] H. Akabori and M. Nakamura, J. Antibiot., Tokyo [A] *12*, 17 (1959).

and their another homologue *iso*-butyropyrrothine along with a polyene (heptaene) and an unidentified antibiotic in the same fermentation broth.

D. S. Bhate, R. K. Hulyalkar, and S. K. Menon

Antibiotics Research Centre, Hindustan Antibiotics Ltd., Pimpri (near Poona, India), July 4, 1960.

Résumé

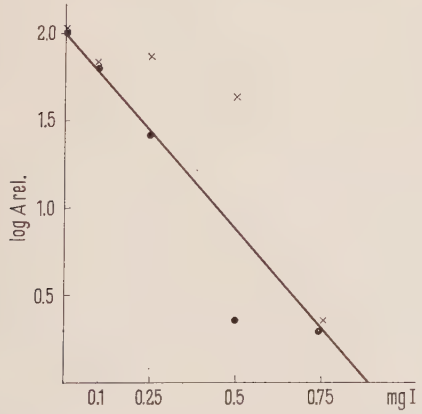
Une nouvelle espèce de Streptomyces (*S. pimprina*) produit l'*iso*-butyropyrrothine avec la thiolutine (l'acétopyrrothine), l'aureothricine (la propriopyrrothine) et un antibiotique antifongique du groupe du polyène (heptaène).

Über die Hemmung von Hurain, einer pflanzlichen Protease, durch Trypsininhibitoren

Hurain ist ein eiweissverdauendes Ferment aus dem Saft von *Hura crepitans* L. (Euphorbiaceae). Es ist von den Papainasen deutlich zu unterscheiden wegen seiner Resistenz gegen Oxydation, weil es von Glutathion und andern Aktivatoren von Papain nicht aktiviert wird und weil es Ascaris und andere Darmparasiten *in vitro* nicht angreift, wie es die Papainasen tun[1].

Aus der letztgenannten Eigenschaft wurde geschlossen, dass Hurain möglicherweise von Trypsininhibitoren in seiner Wirkung gehemmt wird, wie das Trypsin oder Chymotrypsin. In der vorliegenden Arbeit wurde diese Möglichkeit experimentell bewiesen.

Material und Methoden. Hurain wurde aus dem Saft, der durch Einschnitte in den Stamm von *Hura crepitans* gewonnen wird, durch Filtrierung und Lyophilisierung dargestellt. Eine weitere Reinigung wurde in dieser Arbeit nicht vorgenommen. Das verwandte Trypsin war ein kommerzielles Präparat (Biochemical Preparation Inc. 1:150). Der kristallisierte Trypsininhibitor aus Soyabohnen wurde von derselben Firma bezogen, die Inhibitoren aus Ascaris und Bohnen wurden nach den entsprechenden Vorschriften der Literatur hergestellt[2,3]. Die proteolytische Aktivität wurde nach der Methode von Anson[4] mit leichten Modifikationen bestimmt.



Hemmung von Trypsin und Hurain durch Bohnen-Hemmstoff. *Abzisse.* mg des Hemmstoffes. *Ordinate.* Log der Enzymaktivität nach Anson[4]. Jeder Ansatz bestand aus 5.0 ml Substrat, 1.0 ml Ferment (1 mg Trypsin oder 6.66 mg Hurain) und der entsprechenden Menge Hemmstoff. Die Fermentaktivität ohne Hemmstoff wird als 100% bezeichnet. Die Ferment- und Hemmstoffmengen wurden bestimmt als N × 6.25.

Hemmstoff	Hemmung %	
	Trysin	Hurain
—	0	0
Bohnen	26.6	29.5
Ascaris	18.8	18.5
Soya	28.8	34.0

Hemmung von 3 Inhibitoren auf die proteolytische Wirkung von Trypsin und Hurain, ausgedrückt in % der Anfangsaktivität und errechnet pro mg Hemmstoff.

Ergebnisse. In der Fig. wird das Ergebnis der Hemmung von Trypsin (1 mg/Versuch) und Hurain (6,6 mg/Versuch) durch verschiedene Mengen von Bohneninhibitor angegeben. Die Werte beider Fermente fallen unter Berücksichtigung der Streuung auf eine Gerade, wenn die Hemmstoffmengen gegen den Logarithmus der Fermentaktivität aufgetragen werden.

Der Vergleich der 3 geprüften Inhibitoren wird in der Tabelle vorgelegt. Es wurde in jedem Versuch mit 5 verschiedenen Mengen von Ferment und einer festen Menge von Hemmstoff (0.1 mg für Trypsin und 0.25 mg für Hurain) gearbeitet. Die Hemmung ist in % der Anfangsaktivität, die in Trypsineinheiten errechnet wurde, ausgedrückt und auf 1 mg Hemmstoff berechnet. Die Inhibierungswirkung der 3 geprüften Hemmsubstanzen war quantitativ fast gleich stark für beide untersuchte Enzyme.

W. G. Jaffé[5] und Dinah S. de Seidl

Facultád de Ciencias, Universidád Central de Venezuela, Caracas, 10. Mai 1960.

Summary

Hurain, a protease from the sap of the tree *Hura crepitans* L., is inhibited by trypsin inhibitors from soya, kidney beans, and ascaris.

Ein ausführlicherer Bericht über die hier mitgeteilten Versuche wird in Acta Científica Venezolana erscheinen.

[1] W. G. Jaffé, J. biol. Chem. *149*, 1 (1943).
[2] N. M. Green, Biochem. J. *66*, 416 (1957).
[3] D. E. Bowman, Proc. Soc. exp. Biol. Med., N. Y. *86*, 491 (1954).
[4] M. L. Anson, J. gen. Physiol. *22*, 79 (1938/39).
[5] Z. Zt. Max-Planck-Institut für Eiweiss- und Lederforschung, München 15.

Pharmacological Actions of 4-Hydroxytryptamine and 4-Hydroxytryptophan

4-Hydroxytryptamine (4-HT) and 4-hydroxytryptophan(4-HTP) are of conspicuous biological interest because 4-HTP is the only tryptophan amino acid which so far has been found to be decarboxylated in the mammalian organism[1], and because 4-HT, besides being one of the most active hydroxyindolealkylamines, is strictly re-

The amines and amino acids used in the present investigation were synthetized at the Farmitalia S. p. A. Research Laboratories, Milan, by Dr. C. Pasini and V. Coló.

[1] V. Erspamer, A. Glässer, M. B. Nobili, and C. Pasini, Exper. *16*, 506 (1960).

lated to psilocin, a naturally occurring compound, which is nothing but its N,N-dimethyl derivative[2].

The present communication describes some pharmacological actions of 4-HT and 4-HTP, as compared with those of 5-hydroxytryptamine(5-HT) and 5-hydroxytryptophan(5-HTP). Cats were anaesthetized with chloralose and dogs with Pernocton.

4-Hydroxytryptamine and 5-hydroxytryptamine

Blood pressure. In the intact cat, 4-HT (100 μg/kg, i.v.) produced a rise of blood pressure which was 2–6 times less intense but more sustained than that elicited by the same dose of 5-HT. In the spinal cat, both 5-HT und 4-HT provoked hypertension but, whereas with 5-HT the pressure rise was proportional to the dose administered, this did not occur with 4-HT.

Respiration. In the dog, 5-HT(3-4μg/kg and more, i.v.) produced a transitory stimulation of respiration, eventually followed by a period of hypopnoea. 4-HT required a higher dosage than 5-HT and the result was always respiratory depression.

In the cat both 5-HT and 4-HT produced bronchospasm. The effect of 4-HT(100 μg/kg, i.v.) was 2–4 times less intense but lasted longer than that caused by 5-HT.

Dog urinary bladder in situ. The stimulant action of 4-HT was approximately 2 times less intense than that of 5-HT (minimum active dose: 1–2μg/kg, i.v.), but was more sustained.

Diuresis of hydrated rats. Unlike 5-HT, subcutaneous 4-HT showed only a poor antidiuretic effect. In fact, 6 mg/kg were less effective than 0·04 mg/kg 5-HT.

Nictitating membrane. 5-HT was 2–5 times more active than 4-HT in contracting the nictitating membrane of the cat, but whereas the 5-HT contraction was rapidly followed by relaxation, that caused by 4-HT lasted considerably longer.

4-Hydroxytryotophan and 5-hydroxytryptophan

Blood pressure. Intravenous 5-HTP, in doses of 2·5-5 mg/kg, elicited in the cat a moderate fall of blood pressure which lasted 15–20 min; the same dose of 4-HTP produced a more pronounced rise of blood pressure, lasting 30–50 min.

Bronchial musculature. Both 5-HTP and 4-HTP produced a spasm of the bronchial musculature when given i.v. in the cat. However, bronchospasm elicited by 4-HTP was considerably more intense and lasted longer than that produced by 5-HTP.

Dog urinary bladder in situ. The contraction produced by 2–4 mg/kg 4-HTP given i.v. was 3 to 4 times more intense and considerably more sustained than that caused by the same dose of 5-HTP.

Nictitating membrane. Single i.v. injections of 2–5mg/kg 5-HTP did not contract the membrane; the same doses of 4-HTP produced a contraction lasting 30–50 min.

Gastrointestinal tract. In the diarrhoea test in mice, the ED50 of 5-HTP by i.p. route was 25μg/mouse, the ED 100, 300 μg/mouse. For 4-HTP the ED50 could not be exactly established, but it was not less than 400 μg/mouse.

Diuresis of hydrated rats. Like 5-HTP, also 4-HTP caused in hydrated rats an evident antidiuretic effect. 4-HTP was approximately half as potent as 5-HTP, the minimum active i.p. dose being 20 mg/kg. At high dose levels, 100 mg/kg and more, 4-HTP produced renal lesions identical to those seen after 5-HTP.

Body temperature. In mice pretreated with 100 mg/kg iproniazid, 30–60 mg/kg 4-HTP, given i.p., produced, like 5-HTP, a remarkable increase of the rectal temperature (1·5° for 60 mg/kg 4-HTP) accompanied by tremors, lasting 60–90 min. No signs of excitement appeared, but the animals were rather depressed.

In rabbits, similarly pretreated with iproniazid, i.v. doses of 5 mg/kg 4-HTP produced a rise of rectal temperature (1·5–2°) similar to that caused by 15 mg/kg 5-HTP, lasting more than 4 h.

Intraspecific aggressive behaviour of mice. Like 5-HTP, also 4-HTP reduced the aggressive behaviour in male mice. At the i.p. dose of 120 mg/kg, 4-HTP abolished fighting in 50% of the treated couples. The same effect was obtained with 100 mg/kg 5-HTP.

Spontaneous electrical cortical activity of the «midpontine pretrigeminal cat». The slow intracarotid injection of 25–40 mg 4-HTP brought about a synchronization of the EEG, similar in every respect to that produced by 8–20mg 5-HTP or 1–2 mg thiopental.

It may be seen from the preceding data that 4-HT and 4-HTP display in the organism effects which are similar to those possessed by 5-HT and 5-HTP. Both amino acids act presumably after having been decarboxylated to the corresponding amines. However, whereas 5-HT and 5-HTP produce pharmacological effects which, with some important exceptions, are more intense than those caused by 4-HT and 4-HTP, the two 4-hydroxyindoles, and especially 4-HTP, cause more prolonged effects than the corresponding 5-hydroxyindoles. This may in part depend on the fact that 4-HT is a substrate for amine oxidase worse than 5-HT[3].

The failure of 4-HT, in sharp contrast to 4-HTP, to cause antidiuresis is puzzling, but it may depend upon the great lability of the phenolic hydroxy group of 4-HT towards oxidizing enzymes, which could prevent 4-HT from reaching the renal receptors in sufficient amounts or, more likely, for a sufficiently long time, to produce afferent glomerular vasoconstriction.

V. Erspamer, A. Glässer, and P. Mantegazzini

Laboratori Ricerche Farmitalia, Milano and Istituto di Farmacologia dell'Università di Parma (Italy), May 2, 1960.

Riassunto

5-HT e 5-HTP da una parte e 4-HT e 4-HTP dall'altra posseggono su tutti i numerosi reattivi saggiati effetti farmacologici similari i che in generale sono meno intensi ma più duraturi per i derivati 4-idrossiindolici.

[2] A. Hofmann and F. Troxler, Exper. *15*, 101 (1959).

[3] V. Erspamer, R. Ferrini, and A. Glässer, to be published.

The Fate of 4-Hydroxytryptophan in the Rat Organism[1]

Among the many tryptophans so far studied in this Laboratory, DL-4-hydroxytryptophan (4-HTP) was one of the few which were found to be decarboxylated *in vitro* by mammalian decarboxylase[2]. It is probable that 4-HTP is the precursor amino acid of psilocin (N,N-dimethyl-4-hydroxytryptamine) and psilocybin (the O-phosphate ester of psilocin) and, hence, that 4-HTP is an amino acid occurring, together with 5-HTP, in nature. These consid-

A full report on the methods used in this study, as well as on the biochemical and pharmacological results will be published elsewhere, together with the discussion of the collected data.

[1] Supported by a grant from the Rockefeller Foundation, New York.

[2] V. Erspamer, A. Glässer, C. Pasini, and G. Stoppani, Nature, in press.

Urinary 4-Hydroxyindoles in Rats given 4-HTP

Dose and administration route of DL-4-HTP	4-Hydroxyindole content (in mg) of 24 h urine		
	4-HTP	4-HT (base)	4-HIAA (as 4-HTP)
80 mg (100 mg/kg) i. p.	13.4	6.3	25.0
72 mg (100 mg/kg) i. p.	9.6	3.0	25.5
33 mg (50 mg/kg) i. p.	3.6	1.6	10.8
14.5 mg (20 mg/kg) i. p.	2.0	1.0	4.2
6.8 mg (10 mg/kg) i. p.	0.7	0.6	1.2
39 mg (50 mg/kg) i. p.	4.7	3.7	8.2
40 mg (50 mg/kg) i. p.	3.8	2.2	5.4
16 mg (20 mg/kg) i. p.	1.8	1.0	2.0
8.3 mg (10 mg/kg) i. p.	1.1	0.9	1.1
7.8 mg (10 mg/kg) i. p.	0.5	0.5	0.7
180 mg (200 mg/kg) per os	6.7	7.7	45.5
86 mg (100 mg/kg) per os	4.6	3.8	20.6
44 mg (50 mg/kg) per os	2.0	2.1	7.8

erations prompted us to carry out a biochemical and pharmacological study of 4-HTP. This preliminary report describes the fate of the amino acid in the organism of the rat.

DL-4-HTP was given by intraperitoneal and oral routes at different dose levels. The semi-quantitative estimation of 4-HTP, 4-hydroxytryptamine(4-HT) and 4-hydroxyindoleacetic acid(4-HIAA) in urine and acetone tissue extracts was carried out by visual comparison of paper chromatograms obtained with different amounts of the above biological materials and paper chromatograms obtained with different amounts of pure DL-4-HTP and 4-HT creatinine sulphate. The values of 4-HIAA were provisionally expressed in terms of 4-HTP.

The solvent used in the ascending chromatography was the *n*-butanol : acetic acid : water mixture (4 : 1 : 5); the most commonly employed developing reagents were the Heinrich and Schuler's NNCD reagent (2-chloro-4-nitro-1-diazobenzene-α-naphtalene sulphuric acid), a stable diazonium salt[3], and the *p*-dimethylaminobenzaldehyde reagent. Urinary 4-HT was also determined by bioassay, using the rat uterus preparation.

Urine chromatograms showed the presence of unchanged 4-HTP and of at least eight 4-HTP metabolites. Five of them could be identified as 4-HT, 4-HIAA and the O-glucuronides of 4-HTP, 4-HT and 4-HIAA. Among the glucuronides, that of 4-HTP was present only in traces, that of 4-HT in considerable amounts. The identification of the glucuronides was carried out after their hydrolysis by β-glucuronidase.

The accompanying Table presents some quantitative data on the content of 4-HTP, 4-HT and 4-HIAA in the urine collected over a 24 h period.

In all examined rat tissues (gastrointestinal tract, liver, heart, testicles, brain, lung, kidney) unchanged 4-HTP and its main metabolites 4-HT and 4-HIAA could be easily detected by paperchromatography and, as for 4-HT, by bioassay.

1 h after intraperitoneal injection of 100 mg/kg DL-4-HTP to rats pretreated with iproniazid, brain contained 2·5 to 4 μg 4-HT base/g wet tissue, liver 60 to 100 μg, lung 4 to 6 μg, kidney 100 to 120 μg, and testicles 10 to 15 μg.

The following conclusions may be drawn from the above data:

(a) 4-HTP, like 5-HTP, is decarboxylated in the rat organism by several parenchymatous tissues, giving origin to 4-HT. This, in its turn, is attacked by amine oxidase, giving origin to 4-HIAA.

(b) liver and still more kidney decarboxylate 4-HTP with particular intensity.

(c) 4-HTP, again like 5-HTP, passes the blood brain barrier, penetrates into the nervous tissue and is there decarboxylated to 4-HT. Both unchanged 4-HTP and its metabolites 4-HT and 4-HIAA are detectable on chromatograms of brain extracts.

4-HT displays, on isolated organs and in the intact organism, many 5-HT-like actions. Obviously, these actions appear also when 4-HTP, the precursor amino acid of 4-HT, is given. However, in addition to peripheral effects, also central effects make their appearance after 4-HTP[4].

V. Erspamer, A. Glässer,
B. M. Nobili, and C. Pasini

Istituto di Farmacologia, Università di Parma (Italy), June 28, 1960.

Riassunto

Nell'organismo del ratto ha luogo una rapida ed intensa decarbossilazione del 4-idrossitriptofano a 4-idrossitriptamina. Oltre ad essere attaccata dalla monoaminossidasi, con formazione di acido 4-idrossiindolacetico, l'amina viene anche O-coniugata con acido glucuronico. Analogamente al 5-idrossitriptofano, anche il 4-idrossitriptofano valica la barriera ematoencefalica dando luogo a formazione di 4-idrossitriptamina nella compagine del tessuto nervoso.

[3] Sold by Hopkin & Williams Ltd., Chadwell Heath (Essex, England).

[4] V. Erspamer, A. Glässer, and P. Mantegazzini, Exper. *16*, 505 (1960).

Induction of Hepatic Cirrhosis in *Iguana iguana* by 3-Monohydroxycholanic Acid Treatment

Holsti[1] stated that a daily administration to the rabbit of a desiccated whole bile preparation rapidly causes a hepatic cirrhosis. On the strength of later findings, it was suggested that the effect was linked to the activity of bile acids[2], although several of the first tested and commoner variants exhibited only a feeble liverdamaging effect when subjected to the present method[2]. Recently, the same author succeeded in inducing in the rabbit hepatic cirrhosis by gastric instillation of 3-monohydroxycholanic acid or lithocholic acid[3].

It seemed desirable to us to perform a corresponding experiment in a lower vertebrate to establish whether a more general significance is to be attached to this induction process. As experimental material, we chose the reptile *Iguana iguana*.

A daily gastric instillation into 14 males and 17 females of 3 ml of a 0·5% water suspension of the sodium salt of 3-monohydroxycholanic acid (Light & Co., Ltd.) appeared to be highly effective and to induce hepatic cirrhosis within a period of three months. No cases of cirrhosis were observed in the untreated control animals of equal size and life time. By administration of higher concentrations of the sodium salt of 3-monohydroxycholanic acid, a more extensive liver damage was obtained and ultimately death of the animals.

[1] P. Holsti, Acta path. microbiol. Scand. Suppl. 113 (1956).

[2] P. Holsti, Naturwissenschaften *45*, 165 (1958).

[3] P. Holsti, Nature *186*, 250 (1960).

Histologically no massive fat infiltration could be observed in the cirrhotic livers. The liver cells showed a distinct hydropic degeneration, while the ultimate picture was in general that of a finely nodular diffuse cirrhosis. The extent of the necrosis varied with the individual resistance of the animal and the dose administered.

As these results are in good agreement with the data obtained by Holsti[3] in the rabbit, the conclusion is certainly justified that a more general significance should actually be attached to this induction process.

A. Stolk

Histological Laboratory, Free University, Amsterdam (Netherlands), July 18, 1960.

Zusammenfassung

Es stellte sich heraus, dass tägliche Instillation von 3 ml einer 0,5%igen wässerigen Suspension des Natriumsalzes der 3-Monohydroxycholansäure in den Magen von *Iguana iguana* innerhalb dreier Monate Leberzyrrhose hervorrief.

Changes in the Topographical Distribution of Glycogen in the Brain of *Iguana iguana* in Dependence on the Environmental Temperature

Until recently it was assumed that the glycogen of the brain is a rather inert substance, which in the various metabolic processes shows no changes of any importance. Recently it was stated, however, that some functional conditions possess their metabolic analogue in changes in concentration of the glycogen in the brain tissue[1–4]. As the pertaining results were obtained with mammals, we decided to perform corresponding experiments with a lower vertebrate, namely the reptile *Iguana iguana*.

In three groups of *Iguana* males and females, the concentration of the glycogen in the brain tissue was determined in the parts of the brain and that at an environmental temperature of 19.7 ± 0.3°C, 29.6 ± 0.3°C, and 36.8 ± 0.3°C. The animals were killed by decapitation and the concentration of glycogen was determined in the cortex cerebri, the diencephalon, the mesencephalon, the cerebellum, and the medulla oblongata by the method of Kerr[5], with the exception that the reduction compounds were determined according to Nelson's method[6]. See in this connection also the publication of Jakoubek and Svorad[7].

From the results given in the Table, it follows that the drop in environmental temperature results not only in a decrease in concentration of the glycogen in the brain tissue, but even in an increase. Between the values for the male and the female sex, no significant differences were observed. Consequently, in the thermoregulation processes accompanying the decrease in environmental temperature, mainly glycogen quantities of tissue other than that of the brain are used. The drop in environmental temperature appears not to inhibit the synthesis of glycogen in thebrain tissue.

In general, these results correspond with those obtained in the rat[4]. Consequently, the possibility is not precluded that the glycogen metabolism of the brain tissue proceeds in essentially the same way as in mammals. Just as in the rat[4], the topographical determination of the glycogen concentration in the brain tissue points to essential changes of this metabolite in the diencephalon at a decrease in temperature, which under these conditions escapes attention, however, in a rough determination of the glycogen in the whole brain[8].

A. Stolk

Histological Laboratory, Free University, Amsterdam (Netherlands), April 11, 1960.

Zusammenfassung

Es wird über experimentelle Untersuchungen der topographischen Verteilung von Glykogen im Gehirn von *Iguana iguana* in Abhängigkeit von der Umgebungstemperatur berichtet.

Parts of the brain	Sex	Glycogen Values E T 19.7 ± 0.3°C	E T 29.6 ± 0.3°C	E T 36.8 ± 0.3°C
C c	M	86.0 ± 6.1	107.1 ± 5.5	85.5 ± 5.2
	F	84.8 ± 7.2	110.6 ± 7.0	87.9 ± 6.5
D	M	98.6 ± 8.6	170.2 ± 9.2	125.1 ± 7.2
	F	97.2 ± 6.0	165.0 ± 7.1	124.6 ± 9.0
M	M	117.5 ± 7.5	126.1 ± 8.4	113.3 ± 6.4
	F	120.7 ± 5.2	129.3 ± 7.5	115.7 ± 5.3
C	M	86.2 ± 5.1	97.8 ± 7.7	82.4 ± 6.9
	F	80.5 ± 8.3	103.7 ± 6.8	79.0 ± 5.1
M o	M	109.9 ± 9.7	140.2 ± 9.7	107.4 ± 7.8
	F	112.3 ± 9.3	143.8 ± 8.0	110.9 ± 10.7

Changes in glycogen concentration in the brain of *Iguana iguana* at various environmental temperature. The glycogen values are given in mg/100 g fresh brain tissue. For the determination of each values 15 animals were used.

M = male, F = female, C c = cortex cerebri, D = diencephalon, M = mesencephalon, C = cerebellum, M o = medulla oblongata, ET = environmental temperature.

[1] M. R. A. Chance, J. exp. Biol. *30*, 468 (1953).

[2] A. V. Palladine, in Troisième Congr. Internat. de Biochimie (Rapports), Bruxelles (1955).

[3] N. Shimizu and Z. Kubo, J. Neuropath. exp. Neurol. *16*, 40 (1957).

[4] D. Svorad, Science *125*, 156 (1957); A. M. A. Arch. Neurol. Psychiat. *77*, 533 (1957); *Symposium on Hypothermia*, Belgrade (1957); Nature (London) *181*, 775 (1958); Naturwissenschaften *46*, 533 (1959).

[5] S. E. Kerr, J. biol. Chem. *116*, 1 (1936).

[6] N. Nelson, J. biol. Chem. *153*, 375 (1944).

[7] B. Jakoubek and D. Svorad, Pflügers Arch. ges. Physiol. *268*, 444 (1959).

[8] V. Pavlovič, C. R. Soc. Biol., Paris *149*, 2216 (1955).

Einwirkung einiger Carbonylreagentien auf das Virus der Maul- und Klauenseuche (MKS)

Auf der Suche nach Substanzen, die infektiöse Ribonucleinsäure (RNS) tierischer Viren stabilisieren könnten, fanden FRANKLIN und WECKER[1], dass Hydroxylamin die infektiöse RNS aus Mäuseencephalitis-Virus inaktivierte und ebenso das intakte Virus der klassischen Geflügelpest, der Schweineinfluenza und der Pferdeencephalomyelitis (West-Typ). Unter gleichen Versuchsbedingungen konnte aber andererseits zum Beispiel beim Virus der atypischen Geflügelpest (New Castle Disease), des Mumps und des Herpes simplex durch Hydroxylamin keine Einbusse der Virulenz festgestellt werden.

Auf Grund ausgedehnter Untersuchungen schlossen die genannten Autoren, dass Hydroxylamin an der RNS angreift – in welcher Weise blieb ungeklärt – und nicht am Proteinanteil des Virus. Die Befunde vor allem aber, dass bei der Inaktivierung gewisser Viren durch Hydroxylamin die antigenen Eigenschaften, wie die der Komplementbindung und der Hämagglutination, unverändert weiterbestehen blieben, gab zu den hier mitgeteilten Untersuchungen Anlass. Diese sollten feststellen,

a) ob das durch Hydroxylamin inaktivierte Virus auch noch immunitätserzeugende Fähigkeiten aufweist, und

b) ob gegebenenfalls andere Carbonylreagentien vom Typus des Hydroxylamins, wie Hydrazin und Semicarbazid, auch zu dieser spezifischen Wirkung auf das Virus fähig sind.

Bei unseren Versuchen beschränkten wir uns auf das Virus der MKS. Die meisten Versuche wurden mit dem O-Typ ausgeführt, doch kamen bei einigen auch die Typen A und C zur Anwendung, da von vorneherein nicht ausgeschlossen werden konnte, dass das durch Hydroxylamin inaktivierte Virus je nach Typ etwas andere immunogene Eigenschaften (zum Beispiel in qualitativer oder in quantitativer Beziehung) aufweisen könnte.

Für die Bereitung der Virussuspensionen benützten wir Zungenaphthen (Blasendecken) von künstlich mit MKS-Virus infizierten Rindern. Das fein zerkleinerte Material wurde in gepufferter Kochsalzlösung vom pH 7,5 (50 g $Na_2HPO_4 \cdot 2\ H_2O$, 2 g KH_2PO_4 und 144 g NaCl auf 20 l H_2O) aufgenommen und 10 min zentrifugiert (8000 T/min) (1 g Zungenepithelien + 10 ml Kochsalzlösung = Virussuspension $1 \cdot 10^{-1}$).

Methodik der Inaktivierung. Die Carbonylreagentien wurden als Hydrochloride eingewogen und jeweils unter Zusatz von Natronlauge in 2-molare Lösungen vom pH 7,5 gebracht. Diese Lösungen wurden mit dem gleichen Volumen Virussuspension 26 h bei 20° stehengelassen. Da die verwendeten Carbonylreagentien starke Blutgifte darstellen, wurden sie nach vollzogener Inaktivierung durch 24stündige Dialyse gegen Leitungswasser entfernt.

Ergebnisse. Hydroxylamin, nicht aber die beiden andern oben erwähnten Carbonylreagentien, vermochte in der angegebenen Versuchanordnung schon nach 15 min Einwirkungsdauer die Standard-Virussuspension, wenn auch nicht vollständig, so doch grösstenteils, apathogen zu machen. (Die Einwirkungsdauer lässt sich leider nicht scharf bemessen. Man kann lediglich die Reaktionsgeschwindigkeit durch Verdünnen der Versuchslösung mässigen. Wir haben dies durch Hinzufügen des 4fachen Volumens an gepufferter Kochsalzlösung (siehe oben) und anschliessende Dialyse getan.) – Bei 26–40 h dauernder Einwirkung konnte auch mit Hydrazin und Semicarbazid in allen Fällen eine restlose Inaktivierung erzielt werden. Die mit derart inaktiviertem Virus immunisierten Meerschweinchen erwiesen sich ohne Ausnahme als geschützt gegen eine generalisierende Krankheit, wenn sie mit dem homologen Virustyp infiziert wurden, während es bei den Kontrolltieren in rund 75% der Fälle zu einer generalisierenden MKS kam[2]. – Die erzeugte Immunität ist streng typspezifisch, das heisst Meerschweinchen, die mit inaktiviertem C-Virus immunisiert worden waren, erkrankten ohne Ausnahme an einer generalisierenden MKS nach Infektion mit dem Virus des O-Typs. – Bei der Mehrzahl der Meerschweinchen konnte schon nach zweimaliger subkutaner Injektion mit inaktiviertem Virus eine Immunität hervorgerufen werden, während eine einmalige Injektion nur eine Teilimmunität hervorzurufen vermochte. Als ganz ungenügend zur Immunisierung erwies sich eine einmalige intrakutane Applikation des inaktivierten Virus in die Planten. Dies dürfte darauf zurückzuführen sein, dass bei dieser Art der Impfung jeweils nur sehr kleine Mengen des Antigens inkorporiert werden können. In der Tabelle ist ein typisches Beispiel unserer Inaktivierungs- und Immunisierungsversuche wiedergegeben.

Am 3. 12., 12. 12. und am 16. 12. 59 wurden den Meerschweinchen Nr. 16071 – 16081 je 0,5 ml mit Hydrazin inaktiviertes MKS-Virus, Typ C, in die Kniefalte eines Hinterbeins injiziert. Am 20. 12. wurden sämtliche Tiere inklusive die Kontrolltiere Nr. 16082 bis 16086 mit virulentem Virus, Typ C (Konzentration $0{,}2 \cdot 10^{-2}$) in beide Hinterpfoten intrakutan infiziert.	Nr. des Tieres	Resultat der Infektion nach			
		1. Tag	2. Tag	3. Tag	4. Tag
	16071	○	○	⌄	↘
	16072	○	○	○	○
	16073	○	○	↘	↘
	16074	○	○	○	○
	16075	○	○	○	○
	16076	○	○	○	○
	16077	○	○	○	○
	16078	○	○	○	○
	16079	○	○	↘	↘
	16080	○	○	○	○
	16081	○	○	○	○
	16082	⌄	⌄	⌄	⌄
	16083	⌄	⌄	⌄	⌄
	16084	⌄	⌄	⌄	⌄
	16085	↘	↘	↘	⌄
	16086	⌄	↘	↘	↘

○ = Keine Erscheinungen von MKS
↘ = Primäraphthe hinten links
⌄ = Primäraphthen hinten links und rechts
| | = Sekundäraphthen vorne links und rechts
⌄ = vollständige Generalisation (hinten 2, vorne 2, Zunge)

Diskussion. FRAENKEL-CONRAT *et al.*[3] haben in jüngster Zeit die Ansicht geäussert, dass dasjenige Virus-Antigen zur Erzeugung einer Immunität als das idealste zu bezeichnen wäre, das bei völlig intaktem Proteinanteil inaktivierte RNS enthält. Es hatte sich nämlich ergeben, dass der von der RNS abgetrennte Proteinteil des Tabakmosaikvirus (TMV) zum Beispiel wenn er dissoziiert ist, eine neue Antigenkomponente manifest werden lässt und sich dann serologisch vom TMV selbst oder dessen intaktem bzw. reaggregiertem Proteinanteil unterscheidet[4]. Unter physiologischen Bedingungen dissoziiert das von der RNS separierte Virusprotein, womit sich dieses für Immunisierungszwecke als ungeeignet erweist.

Herrn Dr. G. A. MOOSBRUGGER, Leiter des Eidg. Vakzine-Instituts in Basel, danken wir, dass er uns die Durchführung dieser Untersuchungen ermöglicht hat.

[1] R. M. FRANKLIN und E. WECKER, Nature *184*, 343 (1959).

[2] Über analoge Versuche beim Rind wird später berichtet.

[3] H. FRAENKEL-CONRAT, M. STAEHELIN und L. V. CRAWFORD, Proc. Soc. exp. Biol. Med. *102*, 118 (1959).

[4] H. G. AACH, Biochim. biophys. Acta *32*, 140 (1959).

Nach den Ergebnissen der vorliegenden Arbeit scheint der Proteinteil des MKS-Virus nicht stark verändert worden zu sein, denn das durch die oben genannten Carbonylreagentien inaktivierte Virus verhält sich bei der Komplementbindungsreaktion wie ein unbehandeltes, infektiöses Virus. Wo der Angriffspunkt dieser Reagentien liegt, lässt sich nicht mit Bestimmtheit sagen. Er dürfte aber auch, hier schon auf Grund der Komplementbindungsreaktion im RNS-Anteil zu suchen sein, wie dies Franklin & Wecker[1] für das Virus der Mäuseencephalitis sehr wahrscheinlich gemacht haben. Ein Vergleich mit dem durch Formalin behandelten MKS-Virus zeigt, dass dieses einen erheblich niedrigeren Antigentiter aufweist als das durch Hydroxylamin inaktivierte Virus.

V. Spuhler und K. Meyer

Eidgenössisches Vakzine-Institut, Basel und Pharmazeutische Anstalt der Universität Basel, 12. Juli 1960.

Summary

The virus of foot and mouth disease is inactivated by hydroxylamine, hydrazine and semicarbazide. Such inactivated virus does not differ serologically from intact virus, and represents an interesting antigen for purposes of immunization.

Glicolisi anaerobia del pancreas di cavia

In precedenti lavori alcuni autori (Schneider *et al.*[1]) hanno studiato le caratteristiche dei mitocondri e dei granuli di secrezione del pancreas; recenti ricerche (Novelli[2]) con il metodo della centrifugazione frazionata hanno permesso di separare nell'omogenato di pancreas due frazioni distinte costituite da granuli di secrezione e da mitocondri nettamente differenziabili. Nella presente ricerca abbiamo studiato il comportamento della glicolisi anaerobia nelle diverse frazioni cellulari del pancreas: granuli di tripsinogeno, mitocondri, fase solubile.

Furono usate cavie del peso di 300–350 *g*, alimentate con dieta a base di crusca e verdura. Gli animali vennero sacrificati mediante dissanguamento ed il pancreas venne rapidamente asportato, lavato in soluzione di saccarosio 0.25 *M* e quindi omogenizzato nell'apparecchio di Potter-Elvehjem. L'omogenato venne centrifugato in centrifuga Servall SS-1 alla temperatura di + 2°C per 6 min a 1000 × *g*, in modo da allontanare i nuclei ed i detriti cellulari. Con una successiva centrifugazione a 2000 × *g* per 10 min, si sedimentarono i granuli di secrezione (frazione P 1); il sopranatante venne centrifugato a 15000 × *g* per 17 min onde ottenere i mitocondri (frazione P 2); la terza frazione (S) era costituita dalla fase solubile. I mitocondri ed i granuli furono risospesi in saccarosio 0.25 *M*, fino a riottenere il volume originario dell'omogenato. La determinazione dell'attività glicolitica venne effettuata secondo il metodo manometrico di Le Page[3]; la prima serie di vaschette conteneva la frazione in esperimento, in soluzione di Le Page, senza aggiunta di alcun nucleotide; nella seconda serie si aggiunsero 0.10 ml di ATP 0.01 *M*; nella terza serie 0.20 ml di DPN 0.003 *M*; nella quarta serie ATP e DPN nelle concentrazioni indicate. L'azoto di ogni frazione usata venne determinato con microkjeldahl. La purezza di ogni frazione venne controllata al microscopio in contrasto di fase (Zeiss Winkel). I valori furono espressi come $QCO_2(N)$, dividendo i µl di CO_2 sviluppati in 60 min/i mg di N delle diverse frazioni.

Dall'esame dei dati esposti nella Tabella, risulta che l'attività glicolitica è più evidente nella frazione P 1 che nella frazione P2. Tale attività è risultata assente nella frazione S. L'aggiunta di ATP e di DPN, separatamente, alla frazione P1 non modifica sensibilmente il $QCO_2(N)$; l'aggiunta, invece, di entrambi i nucleotidi determina un'evidente stimolazione di tale attività. Sulla frazione P2, invece, sia l'ATP che il DPN provocano un aumento dell'attività glicolitica, maggiore per il primo che per il secondo; la miscela dei due provoca un aumento ancora maggiore. Risulta quindi, dai dati sopra esposti, che nella cellula pancreatica gli enzimi della glicolisi anaerobia sono presenti sia nei granuli che nei mitocondri, mentre non è stato possibile evidenziarli nella fase solubile. Il $QCO_2(N)$ della frazione P1 risultò immodificato anche in seguito a ripetuti lavaggi di detta frazione con soluzione di saccarosio 0.25 *M*.

È noto dalle ricerche di numerosi autori: (Le Page e Schneider[4], Kennedy e Lehninger[5], Hers *et al.*[6] che nel fegato, nel rene, nel miocardio, gli enzimi della glicolisi anaerobia sono presenti soprattutto nella fase solubile. Nel caso del cervello, invece, gli enzimi della glicolisi anaerobia sarebbero presenti nei mitocondri (Gallagher *et al.*[7], Abood *et al.*[8]).

In base ai dati ottenuti è necessario ammettere nei granuli e nei mitocondri di pancreas di cavia l'esistenza di condizioni tali da giustificare un comportamento diverso da quello che si verifica nel caso del fegato, del rene e del miocardio.

Glicolisi anaerobia nelle frazioni cellulari del pancreas di cavia. I valori sono espressi come $QCO_2(N)$. ± = deviazione standard.

Aggiunte	Nessuna	ATP	Stim.%	DPN	Stim.%	ATP + DPN	Stim.%
Frazione P1	29.8 ± 10.1	30.9 ± 6.4	3.7	30.7 ± 4.1	3.0	35.7 ± 6.4	19.8
Frazione P2	23.0 ± 9.0	26.6 ± 8.7	15.6	24.6 ± 8.4	6.9	28.4 ± 10.1	23.4
Frazione S	0	0		0		0	
N° esperimenti	6	6		6		6	

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[2] A. Novelli, Relaz. II[a] Congr. Soc. it. Istochimica, Firenze, ott. (1959), in presse.

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[4] G. H. Le Page e W. C. Schneider, J. biol. Chem. *176*, 1021 (1948).

[5] E. P. Kennedy e H. L. Lehninger, J. biol. Chem. *179*, 957 (1949).

[6] H. G. Hers, J. Berthet, L. Berthet e C. de Duve, Bull. Soc. Chim. biol. *33*, 21 (1951).

[7] C. H. Gallagher, J. D. Judah e K. R. Rees, Biochem. J. *62*, 436 (1956).

[8] L. G. Abood, E. Brunngraber e M. Taylor, J. biol. Chem. *234*, 1307 (1959).

La presenza degli enzimi della glicolisi anaerobia sia nei mitocondri che nei granuli di pancreas, permette di formulare l'ipotesi che, accanto ad un'affinità morfologica (Novelli[2]), esista anche una certa affinità funzionale tra granuli e mitocondri.

U. M. Marinari e G. Nanni

Istituto di Patologia generale dell'Università di Genova (Italia), il 11 Aprile, 1960.

Summary

The enzymes of anaerobic glycolysis are present in the granules of secretion and in the mitochondria of pancreas of guinea pig. On the otherhand, it has not been possible to demonstrate their presence in the soluble phase of the cell. Simultaneous addition of ATP and DPN causes an increase of this activity in both fractions; separate addition of these substances causes an increased activity only in the mitochondrial fraction.

Study of the Relationship Between the Magnitude of Biological Activity and the Structure of Aliphatic Compounds

In the literature many papers were published dealing with the subject given in the title of this communication. The aim of these efforts was to find a relation between the magnitude of the biological activity and various physicochemical constants[1–10] (e. g. solubility, surface tension, parachor, tension of saturated vapors, partition coefficient).

The object of our task are compounds of the type (I)

$$R - X$$
(I)

In the formula (I) R denotes an aliphatic substituent (see Table I) and X means a functional group (e. g. $-OH$, $-COO^{(-)}$, $-NH_3^{(+)}$, $-NH.CSS^{(-)}$).

On the basis of our experiments, and of literature data, we suppose that the following equation is tenable:

$$\log (\tau_i/\tau_{Et}) = \alpha\beta. \qquad (1)$$

In Equation (1) τ_i denotes the magnitude of biological activity of an *i-th* member of homologous series, τ_{Et} is a similar quantity corresponding to the reference compound (usually ethyl derivative), α and β are constants. The constant β, which we call the constant of biological activity of a substituent group, is a quantity describing properties of a substituent R, and its value neither depends on the nature of the functional group [see formula (I)] nor on the biological object used. On the contrary, the constant α characterizes the given biological system (compounds of homologous series together with the biological object).

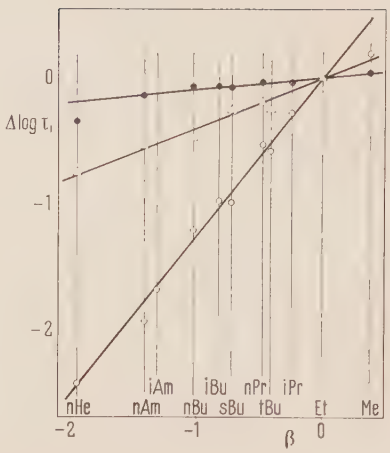

Fig. 1. Dependence of relative molar concentrations of alcohols (○), alkylaminehydrochlorides (Δ), and alkylxanthogenates (●) inhibiting the movement of worms *T. tubifex*[13] on constants β. Abbreviations cf. Table.

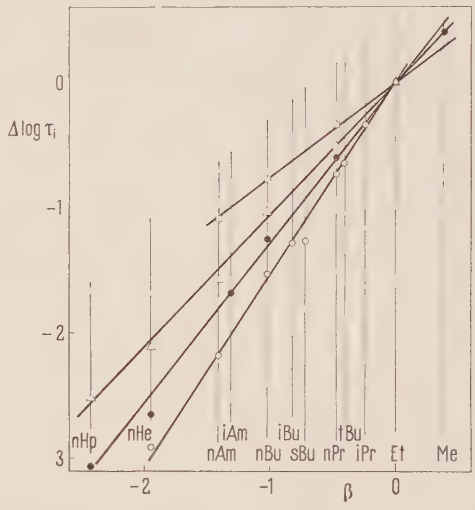

Fig. 2. Dependence of relative molar concentrations of alcohols producing narcosis in red spider[14] (□), *B. typhosis*[15] [(Δ), bactericidal concentration], tadpoles[16] (●), and fishes *Lebistes reticulatus*[13] (○) on constants β. Abbreviations cf. Table.

Values of constants β for substituents R

	Substituent (R)	β		Substituent (R)	β
Me	Methyl	0.38	*n*Bu	*n*-Butyl	−1.02
Et	Ethyl	0.00	*n*Am	*n*-Amyl	−1.41
iPr	isoPropyl	−0.24	*n*He	*n*-Hexyl	−1.95[a]
tBu	tert. Butyl	−0.41	*n*Hp	*n*-Heptyl	−2.43[a]
*n*Pr	*n*-Propyl	−0.47	*n*Oc	*n*-Octyl	−2.92[a]
iBu	isoButyl	−0.82	*n*No	*n*-Nonyl	−3.46[a]
neole	neoPentyl	−0.95	*n*De	*n*-Decyl	−3.80[a]

[a] These values were determined with the aid of data presented in papers[11,12].

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[14] W. H. Read, Ann. appl. Biol. *19*, 432 (1932).
[15] F. Tillex and J. Schaffer, J. Bacteriol. *12*, 303 (1926).
[16] H. M. Vernon, J. Physiol. *47*, 15 (1913–1914).
[17] L. P. Hammett, *Physical Organic Chemistry* (McGraw-Hill, New York 1940), p. 184.
[18] R. W. Taft, Jr., J. Amer. chem. Soc. *75*, 4231 (1953).

Equation (1) is formally identical with the equations expressing the linear free-energy relationship (Hammett's[17] and Taft's[18] equations). This simple two-parameter equation is of rather general validity as far as the so-called physical toxicities of compounds of the type (I) are concerned and permits some new views on the question under consideration.

The values of the constants β were determined in such a way that for the selected biological system [LD_{50} of alcohols (i. v.) on white mice] we put the constant α equal to unity, and then the values of log (τ_i/τ_{Et}) obviously present the values of the constants β (Table). In the treatment of the experimental data according to Equation (1) we plot the logarithm of the relative magnitude of biological activity against the value of the constants β. The slope of the resulting straight line indicates the magnitude of the constant α. Equation (1) obviously expresses the correlation between the magnitude of the *relative* activity [log (τ_i/τ_{Et})] and the constant β. We have found that Equation (1) is satisfied not only for homologous series with different functional groups (Fig. 1) but also for different biological objects (Fig. 2).

At the present time we are dealing with the interpretation of the significance of the constants α from the biological point of view and with the question whether Equation (1) is also satisfied by aromatic compounds. Details concerning this paper will be published elsewhere.

R. Zahradník and M. Chvapil

Institute of Industrial Hygiene and Occupational Diseases, Prague (CSR), April 4, 1960.

Zusammenfassung

Es wurde festgestellt, dass die experimentellen Daten der sogenannten physikalischen Toxizität von aliphatischen Verbindungen die Gleichung log $(\tau_i/\tau_{Ät}) = \alpha\beta$ erfüllen, wo τ_i und $\tau_{Ät}$ die Grösse der biologischen Wirkung des i-ten Derivats und des Äthylderivats bezeichnen und α und β die Konstanten sind. Die Bedeutung der beiden Konstanten wurde erwähnt.

L'action empêchante de la γ-globuline standard sur le développement de l'infection syphilitique expérimentale chez le lapin

I. Dans une première série de recherches, nous avons établi l'action empêchante que la γ-globuline standard exerce sur le développement de la syphilis chez des lapins *déjà inoculés* par des implants sous-scrotaux (souche Nichols). Pour obtenir ce résultat, il est nécessaire que les animaux en expérience soient soumis, immédiatement après l'inoculation, à des injections quotidiennes de 2,5 ml de γ-globuline par kilocorps, le nombre total des injections étant de 10[1].

Les animaux traités de cette façon ne présentaient en effet aucune lésion au lieu d'inoculation sauf, entre le 16e et 20e jour, le développement d'un petit nodule, de la grandeur d'un grain de maïs, qui finissait par se résorber assez vite. L'examen ultramicroscopique de cette formation, ainsi que son examen microscopique, ne montraient pas de tréponèmes.

Les passages de contrôle intra-testiculaires chez d'autres lapins, tant avec le produit en question qu'avec des ganglions inguinaux, sont tous restés négatifs. Ce fait démontre que le petit nodule passager sous-testiculaire ne représentait qu'une réaction tissullaire locale envers des corps tréponémiques détruits. La sérologie s'est montrée négative, elle ne présentait qu'un faible crochet de positivation des réactions de flocculation seulement, d'une durée de 7 jours, vers la 6e semaine, phénomène dû probablement à une excitation antigénique passagère en rapport avec les désintégrations tréponémiques de l'inoculum, phénomène comparable à celui décrit par Gastinel[2]. La γ-globuline conférait aux animaux en expérience une certaine immunité, dont la durée ne dépassait pas 12 semaines. Passé ce délai, les réinoculations devenaient positives, ce qui constituait une preuve de plus que les premières avaient échouées.

II. Dans une seconde série d'expériences, nous avons pu démontrer l'action destructive exercée par la γ-globuline, *in vitro*, sur les tréponèmes.

La trituration préalable de l'inoculum avec cette substance (fragment de syphilome d'environ 75 mm³ délayé dans 1 ml de γ-globuline, maintenu 1 à 2 h à 37°C) et l'inoculation intra-testiculaire de 0.5 cm³ de produit ainsi traité, chez des lapins, est restée également négative, comme dans les expériences précédentes, fait contrôlé ici aussi par des examens histologiques, ultramicroscopiques et sérologiques, de même que par les passages de contrôle négatifs du matériel testiculaire et des ganglions inguinaux.

De ce que nous venons de dire, se dégagent les conclusions suivantes:

a) L'administration de 10 injections quotidiennes de γ-globuline à la dose de 2,5 ml/kilocorps par piqûre, chez les lapins, aussitôt après leur inoculation intra-testiculaire de la syphilis, empêche chez l'animal le développement de l'infection et crée chez lui un état d'immunité durant environ 12 semaines[3].

b) L'empêchement du développement de l'infection expérimentale peut être également obtenu par l'action *in vitro* de la γ-globuline sur les tréponèmes de l'inoculum.

S. Gh. Nicolau et Al. Badanoiu

Section dermatologique de l'Académie des Sciences, Boucarest (Roumanie), le 25 février 1960.

Summary

Standard γ-globulin injections inhibit the development of the syphilitic experimental infection in rabbits infected with Nichols stem. The same results may be obtained by adding γ-globulin to the inoculum.

[1] Des doses moindres de γ-globuline ne produisent qu'un retard du développement du chancre d'inoculation.

[2] P. Gastinel et P. Collart, Ann. Derm. Syph. *83*, 1 (1956).

[3] Chez l'homme nous avons expérimenté dans deux cas l'action exercée par la γ-globuline sur la disparition des tréponèmes des lésions. Dans un premier, atteint de syphilome séro-négatif, les tréponèmes ont disparu après 5 injections de 4 ml chacune. Dans le second, plus ancien, atteint de syphilides vulvaires hypertrophiques, la disparition des tréponèmes a nécessité 11 injections de 4 ml chacune.

Il est difficile de tirer des conclusions d'un nombre aussi restreint de cas, mais en nous tenant aux faits observés, il semble que la γ-globuline exerce, chez l'homme aussi, une action antitréponémique certaine, malgré le caractère réduit des doses employées.

Activation of Fatty Acids in the Liver in Carbon Tetrachloride Poisoning

Recents experiments[1,2] have cast doubt upon the hypothesis that the increase in liver fat, which follows the oral administration of CCl_4, is the result of a lesion in the mitochondria.

Previous work in this laboratory[3] has shown a decrease of fatty acid activation in rat liver during the later stages of carbon tetrachloride intoxication. The present results seem to provide further insight into the mechanisms underlying CCl_4 hepatotoxicity in the initial stages.

Methods. Male rats weighing 180–200 g were used. All food was withdrawn 12 h before CCl_4 feeding. Carbon tetrachloride in a 1:1 mixture with olive oil was introduced into the stomach by intubation at a dose of 0.5 ml of the mixture/100 g of body weight. The animals were killed by cervical section and exsanguinated after 3, 4, 5, 7 h. The liver was homogenized with a teflon homogenizer of the Potter-Elvehjem type at 0°C in 0.25 *M* sucrose solution containing 0.03 *M* $MgCl_2$.

The activation of palmitic acid was tested by the hydroxamic acid method[4–8] without adding CoA, in order to evaluate mainly the initial step (palmityl-AMP) of activation[6,7].

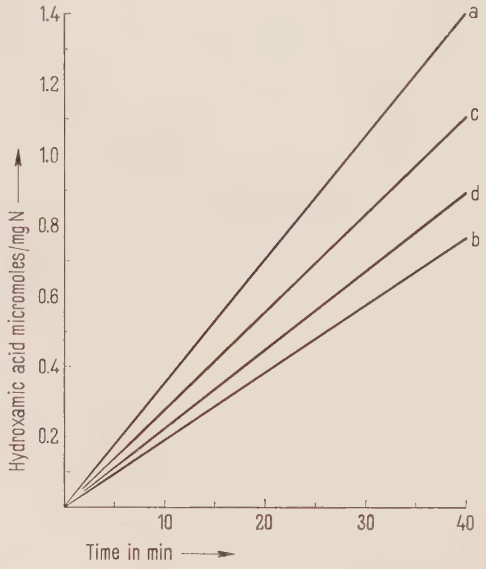
Total liver fat was examined by extraction in a Soxhlet apparatus for 24 h with alcohol-ether mixture (3:1).

The effect of CCl_4 *in vitro* was estimated as follows. For the activation of palmitic acid, the liver from normal rats was homogenized in one volume of 0.25 *M* cold sucrose and diluted to appropriate volumes in 0.25 *M* sucrose saturated or not with CCl_4 (100% saturation is about 0.005 *M*). The final suspension, corresponding to 2 mg of total liver nitrogen, contains 4,3 μM of CCl_4.

For the butyric acid activation, the enzyme was extracted from a liver acetone powder with 10 vol of 0.0128 *M* cold K-phosphate buffer pH 7.7[5,6,9] and diluted to appropiate volumes in the same buffer saturated or not with CCl_4. 1 ml of the extract corresponding to 0.9–1 mg of total nitrogen, contains 4 μM of CCl_4. In this case any formation of hydroxamic acid represents only the initial step of fatty acid activation (acyl-AMP), since the amount of CoA initially present in the extract cannot but be very low[6].

There are many evidences that our experimental conditions permit exclusively the measure of the hydroxamic acid produced by the reaction between *activated* fatty acids and NH_2OH. These evidences are the following:

(1) Lipman and Tuttle[8] showed that the amount of hydroxamic acid formed by liver lipase depends on the length of chain of the fatty acid: it has a maximal level for 8 C and is about zero for 16 C. This fact has been confirmed in our laboratories[4,5].



Influence of CCl_4 *in vitro* on the activation of palmitic and butyric acids. Conditions: see text and notes of the Table.
a = butyric acid μM 10. b = butyric acid μM 10 and CCl_4 μM 1.0. c = palmitic acid μM 2. d = palmitic acid μM 2 and CCl_4 μM 1.1.

	No. of rats	h post CCl_4	Liver fat mg/100 mg dry weight	τ	Significance	Hydroxamic acid μM/mg N	τ	Significance
Controls	6	—	19.8 ± 3.9	2.0	$P < 0.05$	1.03 ± 0.01	0.4	$0.6 < P < 0.7$
CCl_4 fed	6	3	23.8 ± 2.2			1.06 ± 0.05		
Controls	4	—	20.8 ± 2.5	2.4	$P < 0.05$	$1,04 \pm 0.06$	1.6	$0.1 < P < 0.2$
CCl_4 fed	4	4	25.6 ± 2.5			0.96 ± 0.07		
Controls	5	—	19.4 ± 2.9	5.1	$P < 0.01$	1.04 ± 0.09	3.3	$P < 0.01$
CCl_4 fed	5	5	27.7 ± 1.7			0.85 ± 0.09		
Controls	7	—	19.1 ± 2.0	8.9	$P < 0.01$	1.10 ± 0.1	3.6	$P < 0.01$
CCl_4 fed	7	7	28.0 ± 1.5			0.84 ± 0.1		

Activation of palmitic acid in liver after CCl_4 feeding. *Conditions.* Liver homogenate corresponds to 0.5 mg of total nitrogen; time of incubation 40 min; temperature 38°C; pH 7.4. *Medium.* 10 μM of ATP; 30 μM of KF; 750 μM of $NH_2OH \cdot HCl$ (KOH to pH 7.4); 2 μM of palmitic acid (NH_4OH to pH 7.4). Blancs: without substrate and without ATP; Palmityl hydroxamic acid was determined by the method of Lipmann and Tuttle[8].

[1] R. O. Recknagel and D. D. Anthony, J. biol. Chem. *234*, 1053 (1959).
[2] L. Share and R. O. Recknagel, Amer. J. Physiol. *197*, 121 (1959).
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[11] F. Lynen and S. Ochoa, Biochim. biophys. Acta *12*, 299 (1953).
[12] Y. Stein and B. Shapiro, Biochim. biophys. Acta *24*, 197 (1957).
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[14] A. Kornberg and W. E. Pricer, J. biol. Chem. *204*, 329 345 (1953).

(2) Fluoride is known to be a powerful inhibitor of lipase[3,4]; for this reason our experiments have been carried out with fluoride 0.05 *M*.

(3) For short chain fatty acids (butyric), we have extracted the enzymes from a liver acetone powder by phosphate buffer 0.0128 *M* pH 7.7; it was demonstrated that fluoride completely inhibits any lipase activity in such conditions[5].

Apart from the above evidences, we usually have 2 controls in our determinations, i. e. without substrate and without ATP, in order to ascertain that hydroxamic acid formation we measure is merely due to presence of ATP.

Results. The force feeding of CCl_4 resulted in (a) an increase of total liver fat at 3–4 h, according to Recknagel[1] and (b) a diminished activation of palmitic acid which appears to be almost contemporary to the fatty infiltration and in any case is already statistically significant 5 h after poisoning (Table).

CCl_4 added *in vitro* inhibited the activation of palmitic and butyric acid (Fig.).

Discussion. The experimental work reported here supports the view that the impairment in the ability to activate fatty acids is a direct result of CCl_4 hepatotoxicity. From other experiment[5], we have evidence that the activation takes place in the mitochondrial and microsomal fractions[10]. It is well known that an activation is the preliminary operation for fatty acid oxidation and for synthesis of phospholipids and triglycerides[11–14].

The fact that this activation is very precociously impaired shows that an enzymic lesion has set in just in the period when liver fat is rising. This lesion seems to represent one of the points of the pathological sequences correlated to the early increase in liver fat, which intervenes long before any demonstrable mitochondrial damage as defined[1,2] in terms of ATP ase transformation, loss of pyridine-nucleotide-dependant oxidative function, uncoupling of oxidative phosphorylation and impairment in the ability to reaccumulate potassium.

F. Rossi and M. Zatti

Istituto di Patologia generale, Università di Padova (Italy), May 23, 1960.

Zusammenfassung

Bei CCl_4-Vergiftung zeigt die Leber eine vermehrte Aktivierung der Fettsäuren, die sich fast gleichzeitig mit der fettigen Infiltration kundgibt. Die Abnahme der Aktivierung äussert sich bereits 5 h nach der Vergiftung in statistisch signifikanter Weise. Zugabe von CCl_4 hemmt *in vitro* die Aktivierung von Palmitin- und Buttersäure.

The Histochemistry of the Dehydrogenase Systems in the Aortae of Rabbits with Experimental Atherosclerosis

In a previous work[1] we have studied alkaline and acid phosphatases, non-specific esterase and succinic dehydrogenase (the latter enzyme by means of blue tetrazolium and neotetrazolium) in the aortae of atherosclerotic rabbits and rats. Recently developed methods for succinic dehydrogenase[2], DPN-diaphorase[3], TPN-diaphorase[4], and the dehydrogenases linked to the latter based on the use of 2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene)-ditetrazolium chloride (= nitro-BT) as the chromogenic electron acceptor gave the possibility of investigating the above-mentioned enzymes also in the vascular wall.

The present communication deals with results of a histochemical investigation of succinic dehydrogenase, DPN-diaphorase, TPN-diaphorase, lactic dehydrogenase, malic dehydrogenase, glutamic dehydrogenase, and glucoso-6-phosphate dehydrogenase in the aortae of 9 rabbits with cholesterol atheromatosis (produced by addition of 1 g cholesterol to the daily diet for 4 months) and 5 control animals. Directions given by Pearse[5] have been adhered to. The tetrazolium used was nitro BT.

Activity of the enzymes mentioned in normal aortae can be detected chiefly in the muscle cells of media (in form of small granules-mitochondria) in decreasing order as follows: DPN-diaphorase, lactic and malic dehydrogenases, TPN-diaphorase, succinic dehydrogenase; the activity of glutamic and glucoso-6-phosphate dehydrogenases was negligible. The activity of the endothelium and cellular elements of the intima is weaker, and in some cases cannot be detected in the sections at all. In adventicial fibrocytes a more pronounced activity could be ascertained.

In rabbits with experimental atherosclerosis, enzyme activities in media and adventicia had mostly the same localisation and intensity as in the control samples.

An interesting pattern could be observed in the plaques. DPN-diaphorase (Fig. b), lactic dehydrogenase (Fig. c), malic dehydrogenase, and TPN diaphorase are here localised in the macrophages (lipophages), and in the fibrocytes ('Faserzellen' Benninghoff) in the form of small granules penetrating into all cellular processes. Preparations stained with Oil Red O showed that the localisation of enzymes activities were not always identical with the localisation of lipids. In the same elements succinic dehydrogenase (Fig. a) could be detected, but the corresponding activity was much slighter. A minute activity was shown by glucoso-6-phosphate (Fig. f) but only in some of the plaques. Similarly, glutamic dehydrogenase activity (Fig. e) which was the slightest of all the enzyme systems investigated, was weakly positive only in some cases in the surface parts of the plaques. It must be emphasized that the stain intensities of all the enzymes studied showed differences, which were most apparent upon comparing small plaques with large ones. In the small plaques with a small number of lipophages, reaction was very intense in nearly all cellular elements. In the large plaques, where substantial amounts of cholesterol could be detected, activity was only in the lipophages and fibrocytes at the surface and in depth close to the media. In the center of the plaques which showed the most intense reaction for cholesterol, enzyme reactions were negligible (Fig. b). Above the small plaques and in the neighbourhood of some larger plaques, reactions were rather pronounced also in the endothelia. Similar reactions are also shown by plaques on the semilunar valves (Fig. d).

The reported findings of high activity of the dehydrogenase systems in the small plaques present, as well as the previously reported activities of alkaline and acid phosphatases, and non-specific esterase, further evidence

Further work on the dehydrogenase systems in plaques in rabbit, rats, and humans is in progress and will be reported in detail elsewhere.

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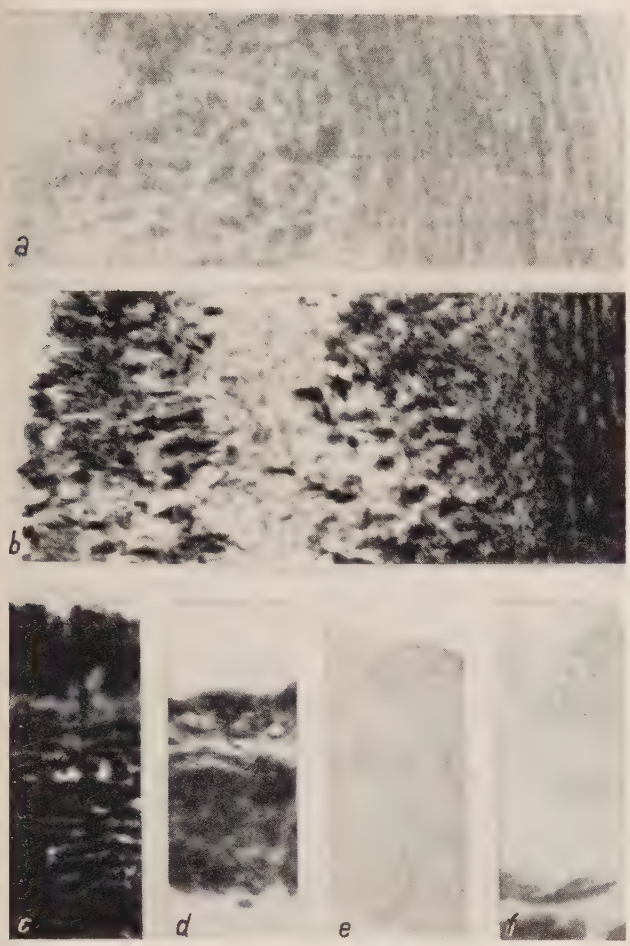

All microphotographies refer to the aortae of atherosclerotic rabbits. a) Succinic dehydrogenase. Slight positive reaction in muscle cells of media (right half of the Figure) and in the macrophages (lipophages) and fibrocytes (left half of the Figure) of intima. b) DPN-diaphorase. Strong positive reaction in macrophages and fibrocytes on surface of the plaque (left quarter) and in muscle cells of media (right quarter). In the center of the plaque (second quarter) negligible reaction. c) Lactic dehydrogenase. Intense reaction in the plaque (top) and in muscle cells of media. d) Lactic dehydrogenase in the plaque (bottom) on the semilunar valve (compare with Fig. c). e) Glutamic dehydrogenase. Reaction is almost negative. f) Glucoso-6-phosphate dehydrogenase. Weakly positive reaction in some cellular elements of the plaque, a stronger one in adjacent myocardium (bottom).

in favour of the concept of relatively high metabolic activity of the intima in the initial stage of the atheromatous lesions.

Z. Lojda and V. Felt

Embryological Institute, Medical Faculty, Charles University and Research Institute for Endocrinology, Prague (CSR). June 15, 1960.

Zusammenfassung

In Aorten von Kaninchen mit experimenteller Atherosklerose wurden die folgenden Dehydrogenasen untersucht: DPN-Diaphorase, Lactat- und Malat-Dehydrogenase, TPN-Diaphorase, Succinat-, Glucoso-6-phosphat- und Glutamat-Dehydrogenase. Die Aktivität der fünf erstgenannten war in den Endothelien, Makrophagen (Lipophagen) und Fibrozyten der verdickten Intima und kleiner atherosklerotischer Beete deutlich erhöht. Die Befunde stehen im Einklang mit der Ansicht, dass die Stoffwechselaktivität der Intima am Anfang der experimentellen Atherogenesis erhöht ist.

The Effect of Litter Rank on the Development of Mouse Lymph Nodes

Evidence has been obtained that maternal age influences the occurrence of mongolism in human beings[1] and that the incidence of spontaneous[2–4] and induced[5] tumors in mice varies with litter rank. The mechanism by which these effects are produced is unknown. The present studies, however, indicate that in young mice of certain strains lymph node size changes significantly in successive litters. Since lymphatic tissue is an important part of host defense, the possibility exists that the influence of litter rank in tumor growth might be mediated through an alteration in lymph node development.

Female mice were used throughout. They were weaned at 5 weeks, thereafter housed 5–12/cage, kept on racks in an air-conditioned room, and fed water, and Purina Laboratory Chow *ad libitum*. At sacrifice, by cervical dislocation, two elbow and two inguinal nodes were removed, trimmed of extraneous tissues, blotted, and the combined weights determined on a Roller-Smith torsion balance.

In the first experiment the lymph node and body weights of 6–7 $^1/_2$ week old second and fourth litter low-tumor C57BL/Sp mice were compared. Mean body and lymph node weights (standard error in parentheses) for 7 second litter mice were 15·9 (0·3) g and 11·6 (0·6) mg respectively, while those for 7 fourth litter mice were 14·0 (0·5) g and 8·3 (0·5) mg respectively. Highly significant ($P < 0{\cdot}01$) decreases in both body and lymph node weights of fourth litter C57BL/Sp mice were observed. On the other hand, similar measurements on first and fourth litter low-tumor $C3H_f$/AnSp mice showed no significant litter rank difference.

In the second experiment (Table I) body and lymph node growth were investigated in C57BL/Sp mice of first through fourth litters by comparing their weights in mice 3 and 6 weeks of age. No significant differences were observed at 3 weeks. Significantly lower body and lymph node weights of mice of the third and fourth litters were found at 6 weeks ($P < 0{\cdot}001$ and $< 0{\cdot}05$ respectively). Smaller percent increases in lymph node weight during the 3–6-week interval were found in mice of second and third litters than in first litter mice. In marked contrast, no such trend was observed in the body growth of successive litters.

Recently[6] it was observed that in foster-nursing mice from strains characterized by large lymph nodes to mothers possessing small nodes the young developed smaller lymph nodes than non-foster-nursed controls. Conversely, foster-nursing mice with normally small nodes to mothers with large lymph nodes resulted in large nodes in the young. These results indicated that a maternal-infant relationship in mice during the nursing period, presumably mediated *via* the milk, is important in lymph node development.

Acknowledgment. This investigation was supported in part by research grant No. C-2151 from the National Cancer Institute, Public Health Service and in part by an institutional grant to the Detroit Institute of Cancer Research from the United Foundation of Greater Detroit through the Michigan Cancer Foundation.

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Tab. I. The effect of litter rank on lymph node development in C57BL/Sp mice[a]

Age weeks	No. of mice	Body weight g	Lymph node weight mg
		First litter	
3	14	6·2 (0·4)	6·0 (0·5)
6	12	15·1 (0·3)	13·9 (1·0)
Increase – %		144	132
		Second litter	
3	26	6·8 (0·3)	6·2 (0·5)
6	27	14·5 (0·3)	13·1 (0·7)
Increase – %		113	111
		Third litter	
3	12	5·8 (0·4)	5·3 (0·7)
6	29	13·1 (0·4)	9·3 (0·7)
Increase – %		126	76
		Fourth litter	
6	9	12·2 (1·2)	9·9 (1·4)

[a] Values are means and their standard errors.

Tab. II. The effect of litter rank on lymph nodes of C3H/AnSp and AKR/Jax mice[a]

Strain	No. of mice	Body weight g	Lymph node weight mg
		First litter	
C3H	12	15·3 (0·5)	21·1 (1·3)
AKR	10	14·1 (0·7)	10·7 (0·6)
		Second litter	
C3H	7	13·2 (0·6)	17·2 (1·1)
AKR	9	14·9 (0·6)	14·6 (1·7)

[a] Values are means and their standard errors.

Work with germ-free animals[7] has indicated that in the absence of an outside stimulus lymphatic tissue fails to develop to an appreciable extent. The present experiments suggest that some maternal stimulus in C57BL/Sp mice apparently diminishes with the age of the mother and/or the number of litters raised.

The effect of litter rank on spontaneous tumor incidence has not been found to be uniform. Bittner[2] observed that mammary tumor incidence was increased in advanced litters. On the other hand, MacDowell *et al.*[4] found a decrease in leukemia incidence in later litters. Preliminary experiments (Table II) indicated that the effect of litter rank on nodes from mice with a high incidence of either spontaneous mammary cancer (C3H/AnSp) or leukemia (AKR/JaxSp) was likewise not uniform. Lymph nodes from 6–$7^1/_2$-week old second litter C3H/AnSp mice were smaller than those of age matched first litter mice; while nodes from 6–$7^1/_2$-week old second litter AKR/JaxSp mice were larger than those of age matched first litter mice. These differences were significant at the 5 percent level. These preliminary findings suggest the possibility that alteration in the quantity of the lymphatic component of host defense may be involved in the alteration of spontaneous tumor incidence by litter seriation.

S. Albert

Detroit Institute of Cancer Research and Wayne State University College of Medicine, Detroit (Michigan), March 21, 1960.

Zusammenfassung

Das Gewicht der Lymphknoten von 6–$7^1/_2$ Wochen alten C57BL- und C3H-Mäusen sank in aufeinanderfolgenden Würfen ab. Umgekehrte Verhältnisse fanden sich bei AKR-Mäusen. Verzögertes Wachstum war für die kleinen Lymphknoten der späteren Würfe bei drei und sechs Wochen alten C57BL-Tieren verantwortlich.

DISPUTANDUM

The Stereochemistry of Reduction by Complex Metal Hydrides

It has been concluded that two effects operate to determine the stereochemistry of an alcohol obtained by hydride reduction of an asymmetric cyclohexanone: the ease of formation of the complex between the carbonyl group and the complex hydride (steric approach control); and the relative energetics of the formation of the products once the initial complex is produced (product development control)[1]. While there is some disagreement over the exact proportions of epimeric alcohols obtained in the reductions[2,3], it seems clear that as Dauben *et al.* originally reported, use of sodium borohydride as reducing agent tends to lead to a greater proportion of the unstable epimeric alcohol than does the use of lithium aluminum hydride. Dauben suggests that this difference in the composition of products is due to the greater effective size of the borohydride species as compared with lithium aluminum hydride.

Although this hypothesis appears to have been generally accepted[4], there are difficulties associated with it. The B-H bond length in borohydride is 1.25 A[5] and it has also been found from measurements of force constants that the Al-H bond length in the aluminum hydride ion is 1.66 A[6]. These bond-length values show that the correctness of Dauben's suggestion depends on the borohydride ion in methanol or pyridine being solvated to a greater extent than the aluminum hydride ion in ether or tetrahydrofuran.

While the theory of ion-solvent interactions is not yet in an entirely satisfactory state, it is thought that monovalent anions which have a crystal radius of 1.80 A or greater are not solvated in water or methanol[7]. The crystal radius of the borohydride ion is 2.03 A[8] and the

We thank Dr. D. J. Royer of the Georgia Institute of Technology, Dr. W. R. Gilkerson of the University of South Carolina, and Dr. R. H. Harris of the University of Nebraska for many helpful discussions concerning the physical and inorganic chemistry of the complex metal hydrides.

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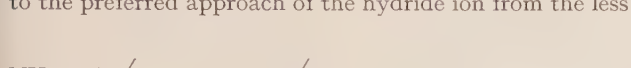
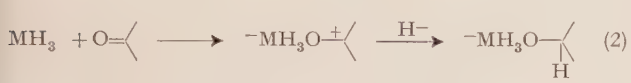
radius of the aluminum hydride ion should be about 0.4 A larger, which suggests that neither the borohydride ion nor the aluminum hydride ion should be solvated. Such experimental evidence as is available tends to support this view, e. g., D'Or and Fuger[9] concluded from studies of infrared spectra that aluminum hydride ions do not complex with ether.

As Dauben's explanation of the difference in amounts of epimeric alcohols obtained by borohydride and aluminum hydride ion reduction is not supported by present solvation theory this alternative is proposed: We suggest that in the reductions of carbonyl groups by complex metal hydrides, two reactions are possible; either a direct attack on the carbonyl by a metal hydride anion (1), giving a relatively large proportion of unstable alcohol, owing to the preferred approach of the hydride ion from the less



(1)



(2)

hindered (equatorial) side of the molecule, or the formation of a complex between the metal hydride and carbonyl (2) which adopts the more stable conformation, and is then reduced. We also suggest that this difference in the course of the reductions is primarily due to the chemical nature of the reducing agent rather than any solvent effects; this is borne out by the results obtained by the reduction of Δ^8-lanosten-3-one (Tab.).

From a consideration of Dauben's hypothesis, one would expect that lithium borohydride in nonpolar solvents would afford about the same or perhaps more equatorial alcohol than does lithium aluminum hydride. However, this does not appear to be the case, for lithium borohydride reduction of lanostenone gives about the same proportion of equatorial isomer as is obtained with the other borohydrides. This similarity of lithium borohydride and the other borohydrides has also been noted in the reduction of tropinone[12]. In addition, lithium tritertiarybutoxyaluminum hydride which is larger than lithium aluminum hydride gives more of the equatorial isomer than does lithium aluminum hydride[10].

It seems likely that lithium aluminum hydride, and the substituted lithium aluminum hydrides, have open to them two modes of dissociation, either a direct ionization[9,13] to lithium ions, and aluminum hydride ions, or a homolytic cleavage to lithium hydride and aluminum hydride (3)[14].

$$LiH + AlH_3 \longleftarrow LiAlH_4 \longrightarrow Li^+ + AlH_4^- \qquad (3)$$

$$MBH_4 \longrightarrow M^+ + BH_4^- \quad M = Li, Na, K \qquad (4)$$

The AlH_3 is capable of behaving as a Lewis acid and may complex with the carbonyl group; the hydride ion is then delivered by any of the appropriate species in solution. The composition of alcohol mixtures obtained by reductions with lithium aluminum hydride varies from the equilibrium mixture obtained using lithium aluminum hydride/aluminum chloride with acetone present[15,16]. This shows that there must be a certain amount of direct attack of AlH_4^- without the preliminary complexing. The greater proportion of stable isomers formed by $LiAl(O\text{-}t\text{-}Bu)_3H$[10] is explained by the tendency of the bulky tritertiarybutoxy aluminum group to take up an equatorial position.

Reducing Agent	% Δ^8-lanosten-3β-ol
$LiAl(O\text{-}t\text{-}Bu)_3H$/tetrahydrofuran[10]	100
$LiAlH_4$/ether[11]	88
$LiAlH_4$/tetrahydrofuran[11]	90
$LiBH_4$/tetrahydrofuran[11]	70
$NaBH_4$/isopropyl alcohol[11]	77
KBH_4/isopropyl alcohol[11]	80

In the case of the considerably more ionic borohydrides[8,17,18] it may be assumed that dissociation occurs to give predominantly metal ion and borohydride ion (4), the direct attack of which will afford more of the unstable epimer than is obtained using lithium aluminum hydride.

In the reduction of a hindered ketone the delivery of hydride from the axial direction is retarded and so a predominance of axial alcohol is obtained when either borohydride or aluminum hydride is used. However, here again the considerations presented above suggest that the percentage of axial alcohol obtained will be greater when borohydride is the reagent.

One special class of hindered ketones involves cases (quoted by Dauben[1]) in which a ketone is reduced by lithium aluminum hydride but not by sodium borohydride. Dauben uses two such examples, methyl b-oxopolyporenate A a-acetate and a 9-bromo-11-oxosteroid, as evidence for his suggestion that the borohydride ion is larger than the aluminum hydride ion. These results can be rationalised in terms of our proposals. Both of the compounds mentioned by Dauben contain not only β-axial methyl groups (which hinder the approach of reagents from the β side) but in addition have large substituents (bromine or methyl) in α-axial orientations. This means that attack on the molecule from the α side is also hindered, and is probably the reason sodium borohydride does not reduce these compounds. In contrast, lithium aluminum hydride can complex with the oxygen atom of the carbonyl group, and thus activates the group so that it attracts in a hydride ion despite the steric hindrance. The hydride ion enters along the less hindered path available; hence, an axial alcohol is obtained.

In addition to explaining the stereochemistry of the products obtained by the reduction of various ketones by metal hydrides, our hypothesis rationalises the differences in reactivity of the various metal hydrides towards certain classes of organic compounds. For example, the well-known selective reduction of ketones in the presence of ester groups by sodium and potassium

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borohydride, while both groups are reduced by lithium aluminum becomes explicable, if one assumes that formation of a carbonyl-metal complex is necessary for the reduction of the less polar ester group.

D. M. S. Wheeler and J. W. Huffman[19]

Department of Chemistry, University of South Carolina, Columbia, and School of Chemistry, Georgia Institute of Technology, Atlanta (U. S. A.), June 20, 1960.

Zusammenfassung

Die gegenwärtig akzeptierte Theorie des Mechanismus der Reduktion von Ketonen mit Metallhydriden wird diskutiert. Verschiedene Unstimmigkeiten werden aufgezeigt, und ein neuer Mechanismus wird vorgeschlagen, der mit den experimentellen Befunden besser übereinstimmt.

Enhancement of Antibody Formation by Hypersensitivity Reaction

It was investigated in young animals whether the formation of antibodies is accelerated or enhanced by injection of endotoxin shortly after birth[1]. Similar results were obtained as in the experiments in which vitamin B_{12} was administered[2]: such pretreatments of the young animals did not affect antibody production but significantly increased their resistance to infection. Attempts were therefore made to elucidate why endotoxins do not influence antibody formation in young animals; injected simultaneously with protein antigens to adult animals, they cause a marked increase in antibody response[3]. We assume that the stimulating action of endotoxin in the adults may be due to the hypersensitivity reaction[4]. This reaction elicited by endotoxin is, however, hardly to be distinguished from its direct toxic action on tissue metabolism. Pure protein antigens were therefore used in order to demonstrate the significance of the hypersensitivity reaction for enhancement of antibody formation.

Rabbits (weight 2–3 kg) were sensitized by injection of 10 mg of human serum albumin (HSA) in lipoid adjuvant into the foot-pads. After four weeks, when high immune response was developed in the sensitized rabbits and intradermal injection of HSA elicited an intense skin test (immediate and delayed type of hypersensitivity were present), the experimental animals were divided in three groups: (1) HSA sensitized animals were given intravenously a challenge dose of HSA (30 μg) simultaneously with a small first dose (10 μg) of ovalbumin (OA). (2) HSA sensitized animals were injected intravenously with ovalbumin (10 μg) only. (3) Normal non-sensitized rabbits received the same amount of HSA (30 μg) + OA (10 μg) as the first group of sensitized animals. The blood from injected animals was collected at intervals of two days and with the sera hemagglutination was carried out using tannic acid treated blood cells coated with HSA or OA[5]. For hemagglutination the micro-method of Takátsy was used[6].

Formation of antibodies to the second antigen – ovalbumin – was demonstrated in the first experimental group, i. e. in the HSA sensitized rabbits which were injected simultaneously with HSA and OA (Fig. 1). The same amount of HSA and OA injected into the control (non-sensitized) rabbits did not give rise to the formation of antibodies to ovalbumin (Fig. 2). That these different

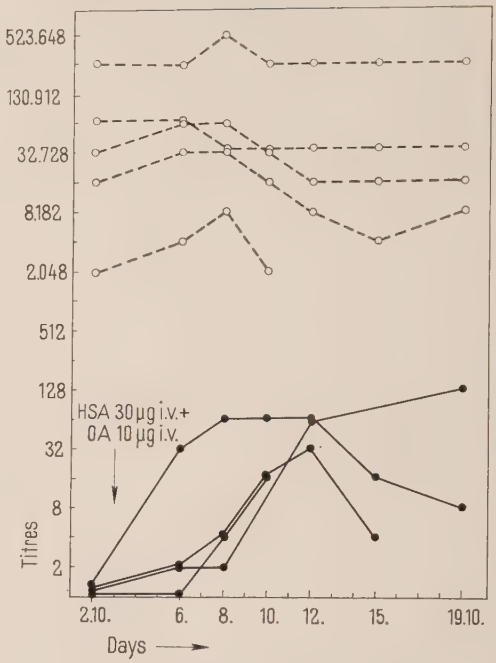

Fig. 1. Antibody titres determined by hemagglutination in the group of HSA sensitized rabbits injected i. v. with 30 μg HSA + 10 μg OA; anti-OA titre – full line, anti-HSA titre – dashed line.

results cannot be explained on the base of secondary response as a cross reaction between HSA and OA is evident from the following findings:

In the rabbits sensitized with HSA and showing anti-HSA titres of thousands, no antibodies to ovalbumin have been demonstrated. When the animal having anti-HSA and anti-OA titre was injected with 30 μg of HSA, only the level of anti-HSA and not anti-OA was affected (Fig. 4). If the HSA sensitized rabbits received injection of OA (10 μg) only, they did not form a demonstrable amount of antibodies to OA (Fig. 3). This also provides evidence that the formation of antibodies to ovalbumin in sensitized animals is not caused by non-specific stimulation of mesenchymal tissue, e. g. by increasing the amount of antibody-forming cells. On the contrary, it may be concluded that stimulation of the antibody response occurred only when the dose of the second antigen (OA) was injected simultaneously with the challenge dose of HSA, i. e. when the hypersensitivity reaction was elicited. Substances released in the course of hypersensitivity reaction might be responsible for the increase in antibody response.

A similar result, i. e. enhancement of antibody formation in sensitized animals, was achieved by Good *et al.*[7]. Animals were sensitized with BCG vaccine and hypersensitivity reaction elicited by injection of old tuberculin.

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[3] A. G. Johnson, J. Gaines, and M. Landy, J. exp. Med. *103*, 225 (1956). – R. M. Condie, S. J. Zak, and R. A. Good, Proc. Soc. exp. Biol. Med., N. Y. *90*, 355 (1955). – P. Kind and A. G. Johnson, J. Immunol. *82*, 415 (1959).

[4] Ch. A. Stetson, J. exp. Med. *101*, 421 (1955).

[5] A. B. Stavitsky, J. Immunol. *72*, 360 (1954).

[6] G. Takátsy, Acta microbiol. Acad. Sci. Hung. *3*, 191 (1955).

[7] R. A. Good, R. M. Condie, G. Thompson, and D. R. Jensen, Fed. Proc. *16*, 178 (1957).

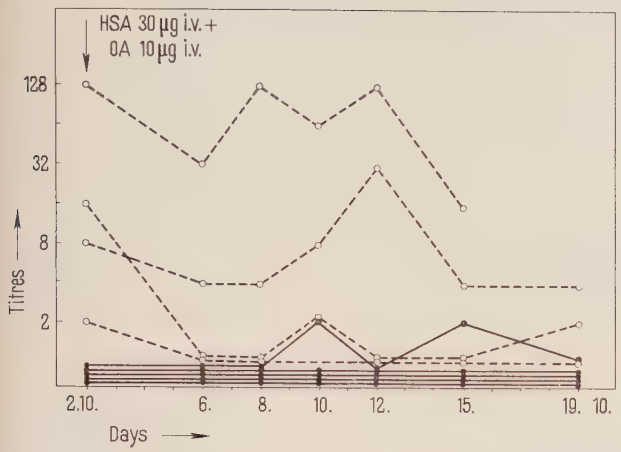


Fig. 2. The group of non-sensitized rabbits injected i. v. with 30 µg HSA + 10 µg OA; anti-OA titre – full line, anti-HSA titre – dashed line.

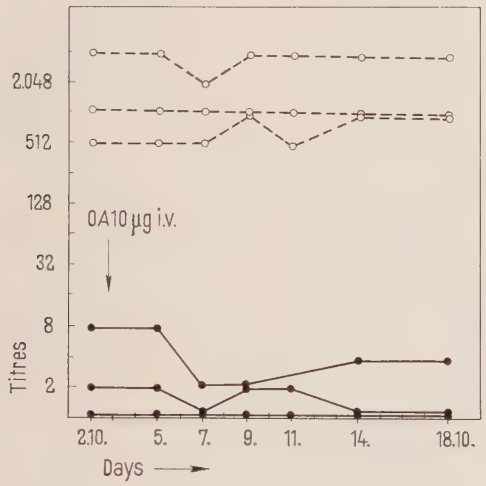


Fig. 3. HSA sensitized rabbits were injected i. v. with 10 µg of OA only; anti-OA titre – full line, anti-HSA titre – dashed line.

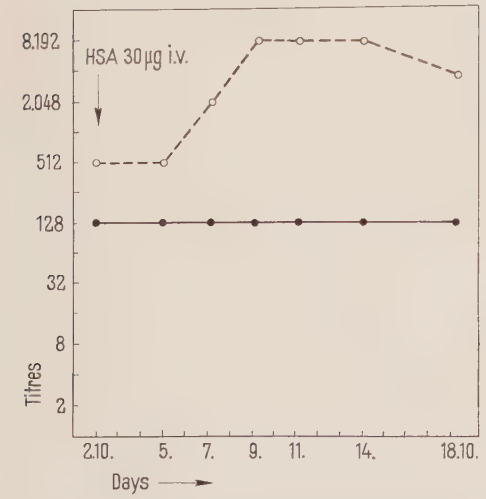


Fig. 4. The rabbit with anti-OA and anti-HSA titre was injected with 30 µg of HSA; anti-OA titre – full line, anti-HSA titre – dashed line.

In that experiment the hypersensitivity might not have been the only cause of enhancement of antibody response since in old tuberculin materials possessing certain of the properties of endotoxin have been demonstrated[8]. Our experiments, in which the hypersensitivity reaction elicited by non-detrimental protein antigens enhanced the antibody response, may support the hypothesis advanced by Stetson[4] that the stimulating action of endotoxin is probably due to the hypersensitivity reaction provoked by this substance. In young animals, therefore, which do not develop hypersensitivity to endotoxin, the antibody response is not enhanced. Another evidence of the absence of hypersensitivity reaction in young animals is the tolerance of significantly higher amount of injected endotoxin than in adults[9].

J. Šterzl

Division of Immunology, Institute of Biology, Czechoslovak Academy of Sciences, Praha, February 25, 1960.

Zusammenfassung

Eine Anzahl Kaninchen wurde durch humanes Serumalbumin (HSA) sensibilisiert. Wurde bei ihnen durch eine weitere Dosis desselben Antigens (HSA) eine Hypersensitivitätsreaktion hervorgerufen, so wurde die Antikörperbildung gegen ein anderes gleichzeitig eingeführtes Antigen (Ovalbumin-OA) stimuliert.

[8] Ch. A. Stetson, S. Schlossman, and B. Benacerraf, Fed. Proc. *17*, 536 (1958).

[9] J. H. Parish and C. C. Okell, J. Path. Bact. *33*, 327 (1930). – P. A. Zahl, S. H. Hutner, and F. S. Cooper, Proc. Soc. exp. Biol. Med., N. Y. *54*, 137 (1943). – L. Thomas, Ann. Rev. Physiol. *16*, 467 (1954).

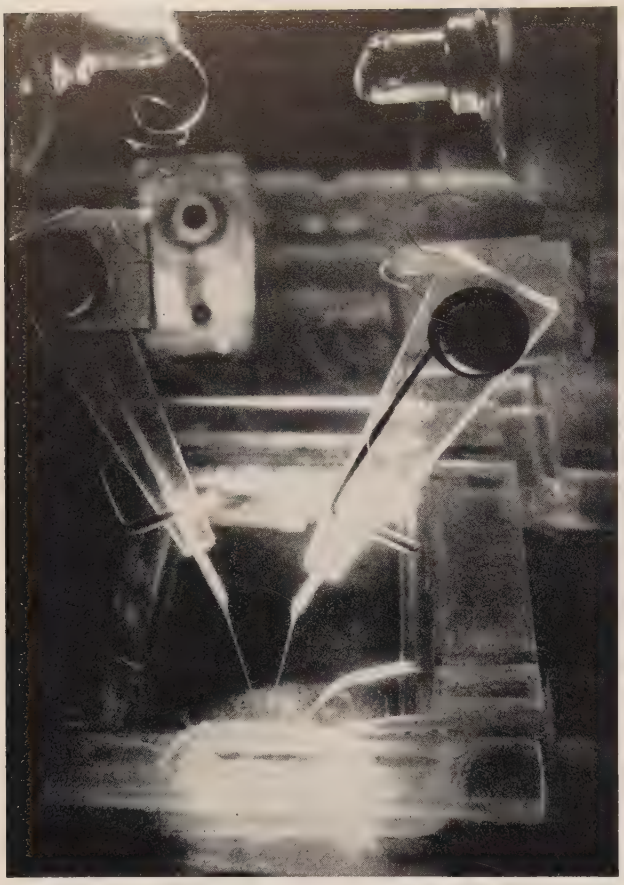
PRO EXPERIMENTIS

Ein einfaches Verfahren zur Sichtbarmachung von Glasmikroelektroden mit Hilfe von Fluorescein

Bei Verwendung von Glasmikroelektroden nach dem bekannten Verfahren von Ling und Gerard[1] zur intrazellulären Ableitung von Ruhe- und Aktionspotentialen ist es meist relativ schwierig, den Einstichort genau zu lokalisieren. Dies ist grösstenteils dadurch bedingt, dass die dünnen, mit 3 *M* KCl-Lösung gefüllten Glaskapillaren nahezu parallel zum Strahlenverlauf des Beobachtungsmikroskopes angeordnet sind; aus diesem Grunde und zum Teil auch wegen Auftretens von störenden Reflexen ist es nicht immer möglich, die Mikroelektroden bis an ihre Spitzen zu verfolgen. Eine Lokalisation des Einstiches ist aber namentlich dann erforderlich, wenn der genaue Abstand zwischen Reiz- und Mikroelektroden bzw. zwischen zwei Mikroelektroden, beispielsweise zur Bestimmung der Leitungsgeschwindigkeit, mit Hilfe eines Okular-Netzmikrometers festgelegt werden soll.

Eine verhältnismässig einfache Methode zur besseren Sichtbarmachung von Glasmikroelektroden sei im folgenden kurz beschrieben: Die mit der Hand oder durch eine Elektroden-Ziehmaschine hergestellten Mikrokapil-

[1] G. Ling and R. W. Gérard, J. cell. comp. Physiol. *34*, 383 (1949).



laren werden im Unterdruckverfahren mit 3 *M* KCl-Lösung unter Zusatz von 2 g Fluorescein-Natrium (Uranin) pro Liter (Aktivierungsmaximum 4600 Å, Fluoreszenzmaximum 5300–5500 Å) gefüllt. Diese Mikroelektroden leuchten im Blaulicht mit gelblich-grüner Fluoreszenz. Bei geeigneter Anordnung des einfallenden Blaulichtstrahles – in unserem Falle wurde in den ausschwenkbaren Filterhalter des Auflichtstrahlenganges (Beleuchtung: 6 V, 30 Watt) eines Zeiss-Epitechnoskop-Mikroskopes ein Blaufilter des Typs Schott BG 25 eingesetzt – können die Mikroelektroden bis an die Spitze genau verfolgt werden (siehe Abb.). Der besondere Vorteil in der Verwendung eines binokulären Auflichtmikroskopes liegt nun darin, dass die in den Mikroelektrodenspitzen auftretende Fluoreszenz von oben her, also nahezu in der optischen Achse des Strahlenganges, beobachtet werden kann. Durch diese besondere Anordnung ist es möglich, eine relativ grosse Schichtdicke der Fluoresceinsäule in der Mikroelektrode zu betrachten, so dass auch verhältnismässig geringe Fluoreszenzintensitäten im Spitzenbereich der Elektrode wahrgenommen werden können.

Eine Beimischung von Fluorescein in der oben angegebenen Konzentration zur 3 *M* KCl-Lösung ändert die elektrischen Eigenschaften dieser Lösung nicht. Der innere Widerstand der so hergestellten Mikroelektroden unterscheidet sich nicht von nichtfluoreszierenden und beträgt nach unseren Messungen für beide Arten von Mikroelektroden zwischen 10 und 50 Megohm. Auch hinsichtlich der von Adrian[2] beschriebenen Tip-Potentiale (in unseren Messungen 2–8 mV) besteht kein Unterschied.

Fluorescein eignet sich zu diesem Zweck besonders, da es 1. eine intensive Fluoreszenz aufweist, 2. in 3 *M* KCl-Lösung in der angegebenen Konzentration gut löslich und stabil ist, 3. die Toxizität dieser Substanz äusserst gering ist (Spector[3]) und 4. die Anwendung solcher Mikroelektroden keinen Einfluss auf das Ruhe- und Aktionspotential ausübt.

B. Pillat und P. Heistracher

Pharmakologisches Institut der Universität Wien, 14. Juli 1960.

Summary

Glass microelectrodes were filled with 3 *M* KCl containing 2 g of sodium fluoresceine/l. The glass-tips of the electrodes are visible under the microscope in a blue activating light.

[2] R. H. Adrian, J. Physiol. *133*, 631 (1956).

[3] W. S. Spector, *Handbook of Toxicology*, Vol. I (W. B. Saunders Co. 1956), p. 142.

CONGRESSUS

The Netherlands

Fifth European Congress on Molecular Spectroscopy

Amsterdam, May 29th to June 3rd 1961

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Please address all correspondence to Dr. D. H. Zijp, Secretary Fifth European Congress on Molecular Spectroscopy, Nieuwe Achtergracht 123, Amsterdam-C (Netherlands).

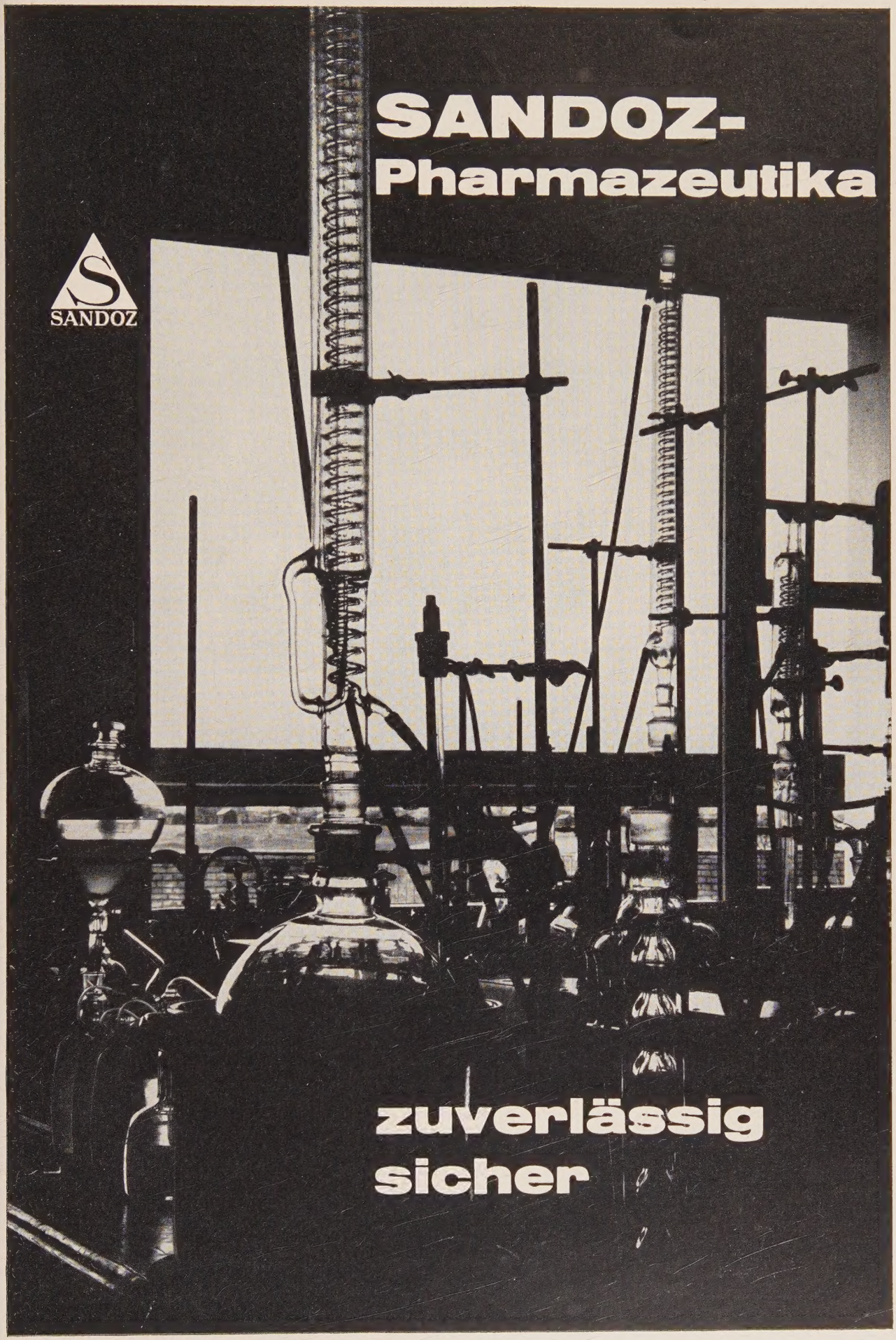
NOTA

U S A

Lectures on Molecular Biophysics

Announcement. Molecular biophysicists are becoming increasingly aware of the biological significance of fast transfer processes utilizing elementary particles (electrons and protons). To inquire into the possible physiological role of fast transfer reactions in eliciting specific interactions in ordered macromolecular structures, a series of seminar lectures was held at the Massachusetts Institute of Technology during the spring term of 1960. Included were lectures on electron and energy transfer mechanisms (Kasha, Taube, Murrell, Weber, Stryer) and on current concepts of proton transport mechanisms (Eigen and Grunwald) of immediate biophysical and biochemical significance. Properties of water and ice crucial for proton transfer were reviewed (Frank, Brady, Klotz, Fernandez-Moran). Six lectures of a more general nature dealt with aspects of macromolecular interaction properties (Gergely, Berendsen, Oncley, Davison, Wiener, Elsässer).

Abstracts of these lectures and pertinent references have been compiled and are available *gratis*. Requests should be sent to Professor F. O. Schmitt, Department of Biology, Massachusetts Institute of Technology, Cambridge 39 (Mass.).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Gratulation der Mitarbeiter der EAWAG
R. Colas, Hommage au professeur Jaag
H. Ambühl, Die praktische Anwendung der elektrochemischen Sauerstoffbestimmung im Wasser, III. Teil
U. d'Ancona, Une invasion d'*Argulus* dans la Lagune de Venise
M. Auerbach, G. Rottengatter, Untersuchungen über den Wasseraustausch der einzelnen Becken des Untersees (I. Teil)
R. Bachofen, Methodische und experimentelle Studie über Stoffwandlungen in Seesedimenten
P. Bourrelly, Une excursion algologique dans la forêt de Fontainebleau
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H. Deuel, P. Dubach, N. C. Mehta, R. Bach, Zur Chemie der organischen Substanz des Bodens
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H. Knöpp, Untersuchungen über das Sauerstoff-Produktions-Potential von Flussplankton
P. Mercier, Les trombes sur le lac Léman
E. Messikommer, Algenflora der Gewässer des St.-Gotthard-Gebietes
C. Merkt, P., Vegetation der alljährlich periodisch trockenliegenden Uferpartien des Sihlsees
L. Minder, Eisen-Mangan-Grundwässer
R. Monod, B. Dussart, Le Léman – I. L'évolution du Léman, vue par le chimiste et le bactériologiste, II. L'évolution du Léman, vue par le biologiste et l'écologiste
F. Nipkow, Zellteilungen bei zwei Desmidiaceen *Micrasterias crux-melitensis* (Ehrb.) Hassalt und *Micrasterias rotata* (Grev.) Ralfs.
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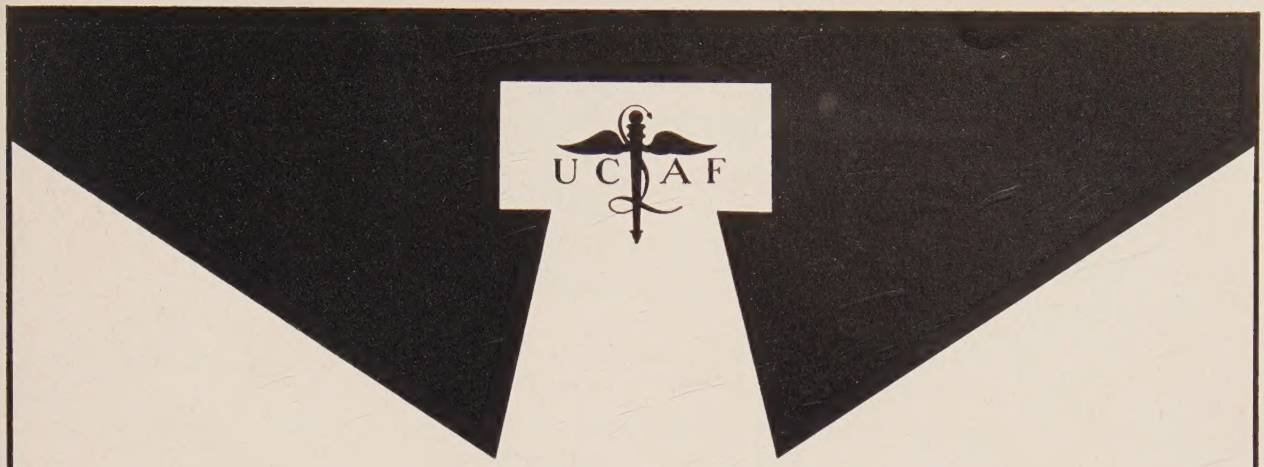

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